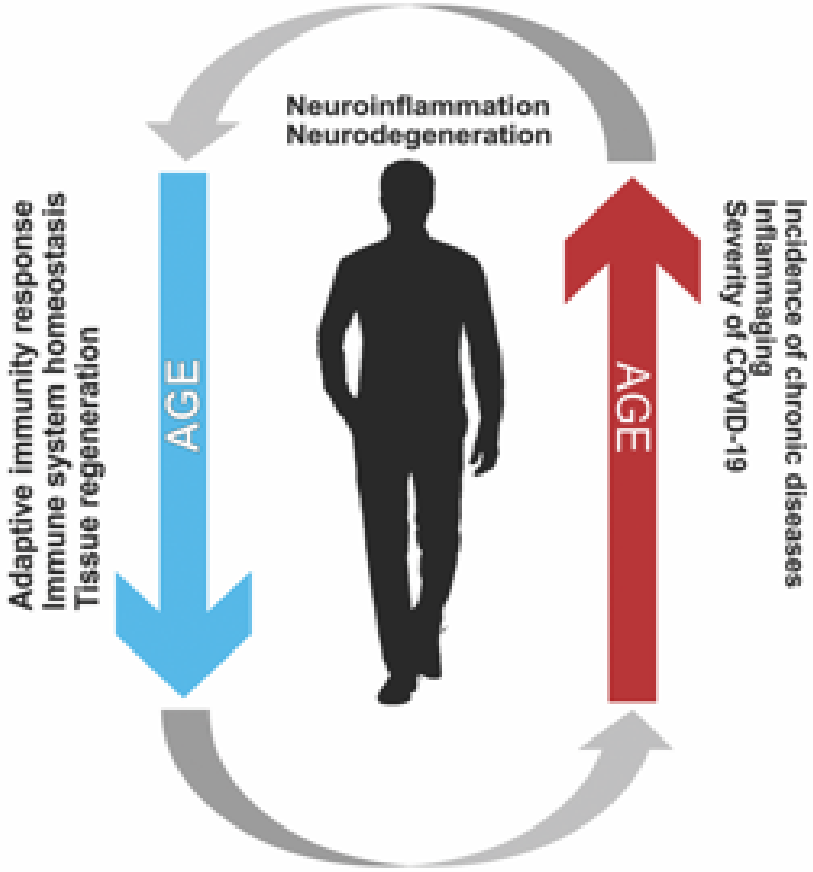

Special Collection on COVID-19

Immunosencescence and inflammaging create a vicious cycle creating an environment favorable for the development of neurodegenerative diseases, see Domingues et al. "SARS-CoV-2, immunosenescence and inflammaging: partners in the COVID-19 crime"

COVID-19 | AGING

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Priority Research Paper

Serum IgM against SARS-CoV-2 correlates with in-hospital mortality in severe/critical patients with COVID-19 in Wuhan, China

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ABSTRACT

Severe/critical patients with coronavirus disease 2019 (COVID-19) have become the central issue in the current global pandemic due to their high mortality rate. However, the relationship between antibody response and clinical outcomes has not been well described in this group. We conducted a single-center, retrospective, cohort study to investigate the relationship between serum immunoglobulin G (IgG) and IgM and clinical outcomes in severe/critical patients with COVID-19. Seventy-nine severe/critical patients with COVID-19 admitted in Wuhan Asia General Hospital in Wuhan, China during January 22, 2020 to March 6, 2020 were included. Serum antibodies were measured at day 25 (SD, 7) post illness onset. The median IgG titer was 113 (IQR 81-167) AU/ml, and IgM titer was 50 (IQR, 23-105) AU/ml. Patients whose IgM titer ≥ 50 AU/ml had higher in-hospital mortality (p=0.026). IgM titer ≥ 50 AU/ml was also correlated with higher incidences of Acute Respiratory Distress Syndrome (ARDS) and sepsis shock. Antibody remeasurements were performed in 42 patients, where IgM titer declined significantly in survivors (p=0.031). Serum IgM titer changes according to the COVID-19 progression. The severe/critical patients with COVID-19 have a higher risk of clinical adverse events when IgM titer ≥ 50 AU/ml. Further decreasing of IgM could imply a better outcome in severe/critical cases.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) was first reported in Wuhan, China in December 2019. The highly contagious pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) soon spread all over the country, and has become a global pandemic [1–4]. Patients infected by SARS-CoV-2 might present from asymptomatic to critical illness with respiratory failure and multi-organ dysfunction, therefore, the disease was categorized into 4 types based on the disease state: mild, moderate, severe, and critical [5, 6]. Severe/critical patients with COVID-19 contributed only 4~15% to overall infected population in different countries [7, 8], however, attentions have been paid to them not only because of their rapid progression in disease, but also due to the greater difficulties in treatment and higher mortality rate [7, 9, 10].

Antibody response in human might be activated at early stage of infectious disease, then be kept stable for a long time. Specific serum immunoglobulin G (IgG) and IgM against SARS-CoV or Middle East Respiratory Syndrome-coronavirus (MERS-CoV) became detectable in patients as early as 11-15 days post illness onset [11, 12]. Similar changes were observed in patients with COVID-19 as IgM and IgG could be detected on 5-14 days after symptom onset [13].

Additionally, the titers of IgM and IgG were significantly correlated with viral load in patients infected by SARS-CoV-2 in a recent finding [14], which promoted the hypothesis that specific antibody against virus might be associated with disease progression in COVID-19. However, reports on clinical profiles of antibody response in severe/critical patients with COVID-19 are scarce.

Hereby, we investigated the serum titers of specific antibodies, IgG and IgM, in severe/critical patients with COVID-19 to explore the association between serum antibody titers and the clinical adverse events in those patients.

RESULTS

Characteristics of the patients

A total of 105 severe/critical patients with COVID-19 admitted to Wuhan Asia General Hospital from 2020.01.22 to 2020.03.06 were enrolled, Of which, 23 were excluded due to the incomplete data, 3 due to negative in antibody measurements. Therefore, 79 patients were reviewed in final analysis, whose mean age was 63 (SD 13) years. Seven (9%) patients were smokers, and comorbidities included 5 (6%) chronic obstructive pulmonary disease (COPD), 31 (39%) hypertension, 13 (16%) diabetes, 6 (8%) coronary artery disease (CAD), and 2 (3%) chronic kidney disease (CKD). The most common symptoms were fever in 64 (81%) patients, cough in 57 (72%), dyspnea in 49 (62%), and fatigue in 44 (56%). The average time from illness onset to admission was 12 days (SD, 6). All patients had significantly change on lung computerized tomography (CT).

Antibody response and in-hospital mortality

Eleven (14%) patients died during hospitalization, who were older than survivors (73 [SD 9] vs 61 [SD 2], *P*=0.002). There were 16 (20%) Acute Respiratory Disease Syndrome (ARDS) and 11 (14%) septic shock happening during hospitalization. Patients had their measurements of serum antibody against SRAS-CoV-2 on day 13 (SD, 7) post admission when tests were available, which was 25 (SD, 7) days after illness onset. The median IgG titer was 113 (IQR, 81-167) AU/ml, and that of IgM was 50 (IQR, 23-105) AU/ml. The difference of IgG titer between survivors and non-survivors was trivial (113 [IQR, 81-167] vs 135 [IQR, 82-158] AU/ml, *P*=0.887), however, IgM titer was significantly increased in non-survivor when comparing with survivors (106 [IQR, 50-128] vs 48 [IQR, 22-84] AU/ml, *P*=0.049) (Figure 1). Forty-two patients had antibody remeasurements 5 (SD, 3) days later. The median IgG titer was 150 (IQR 88-179) AU/ml at 2nd time, and that of IgM was 66 (IQR 32-133) AU/ml. IgG titer remained stable during two measurements in both survivors and non-survivors. Change of IgM titer in survivors showed a significantly decreasing (-4 [IQR -14-0], *P*=0.031), but that in non-survivors didn't show statistical difference (3 [IQR -19-29], *P*= 0.779) (Figure 2).

Serum IgM and clinical outcomes

We further divided patients into two groups using median serum IgM titer as cutoff. Clinical characteristics, such as age, gender, comorbidity, symptoms, time intervals, and vital signs at admission, were similar between the two groups (Table 1). Disease severity was quite different, as a higher incidence of critical cases was seen in the high IgM group (p=0.006) (Table 1). Laboratory measurements presented differently between groups (Table 2). All patients received basic therapy as well as specific treatment based on their disease progression in hospital. More Intensive medical supports were applied in patients whose IgM titer ≥ 50 AU/ml (Table 3).

DISCUSSION

In this retrospective cohort study, IgG and IgM against SARS-CoV-2 in severe/critical patients with COVID-19 were profiled, and relationship between antibody titers

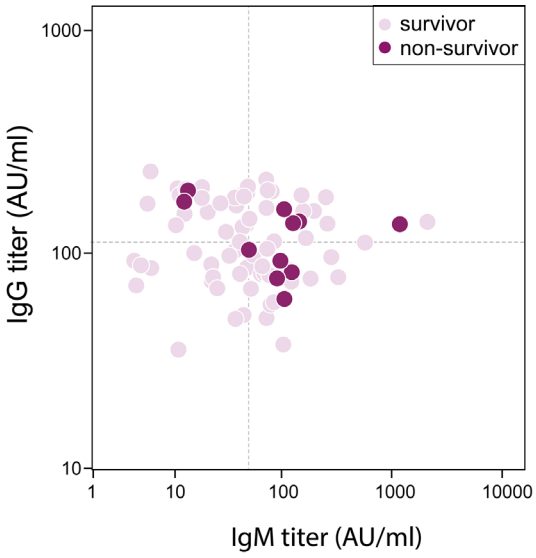

Figure 1 Correlation between Antibody titer and in-hospital mortality in severe/critical patients with COVID-19. Dash lines represent median value as cutoff in IgG (113 AU/ml) and IgM (50 AU/ml) respectively.

and outcomes was also assessed. Specifically, compared with survivors, IgM titer increased in non-survivors while IgG remained unchanged when measurements were performed on 25 (SD, 7) days after illness onset. IgM further decreased in survivors when taking remeasurement 5 (SD, 3) days later. Accompanied by significantly changes in laboratory measurements, more critical cases were seen in patients with IgM titer ≥ 50 AU/ml. Higher frequencies of applying corticosteroids and mechanical ventilation were also observed in patients with IgM titer ≥ 50 AU/ml.

Pneumonia caused by SARS-CoV-2, which was later known as COVID-19, occurred in Wuhan, China in December 2019 [1, 15]. The estimated reproductive number rose from 2.2 to 3.28 [14], and overall mortality rate was around 2-4% [16–18], which might be still increasing as more than one million patients have been confirmed infection, and new deaths are reported globally. Nearly 80% of patients with COVID-19 might present only mild or moderate symptoms, such as fever, and cough [8, 19], however, more than 50% death could be seen in severe/critical cases [7, 20]. Similar to previous studies, non-survivors in our study were older than survivors. There were no differences in comorbidities between survivors and non-survivors in our study, probably due to the variation in the spectrum of underlying diseases. In-hospital mortality (14%) in our study was lower than that in other reports, nonetheless, at least 5 folds higher mortality in severe/critical patients, again, strengthened that great efforts should be paid on this group.

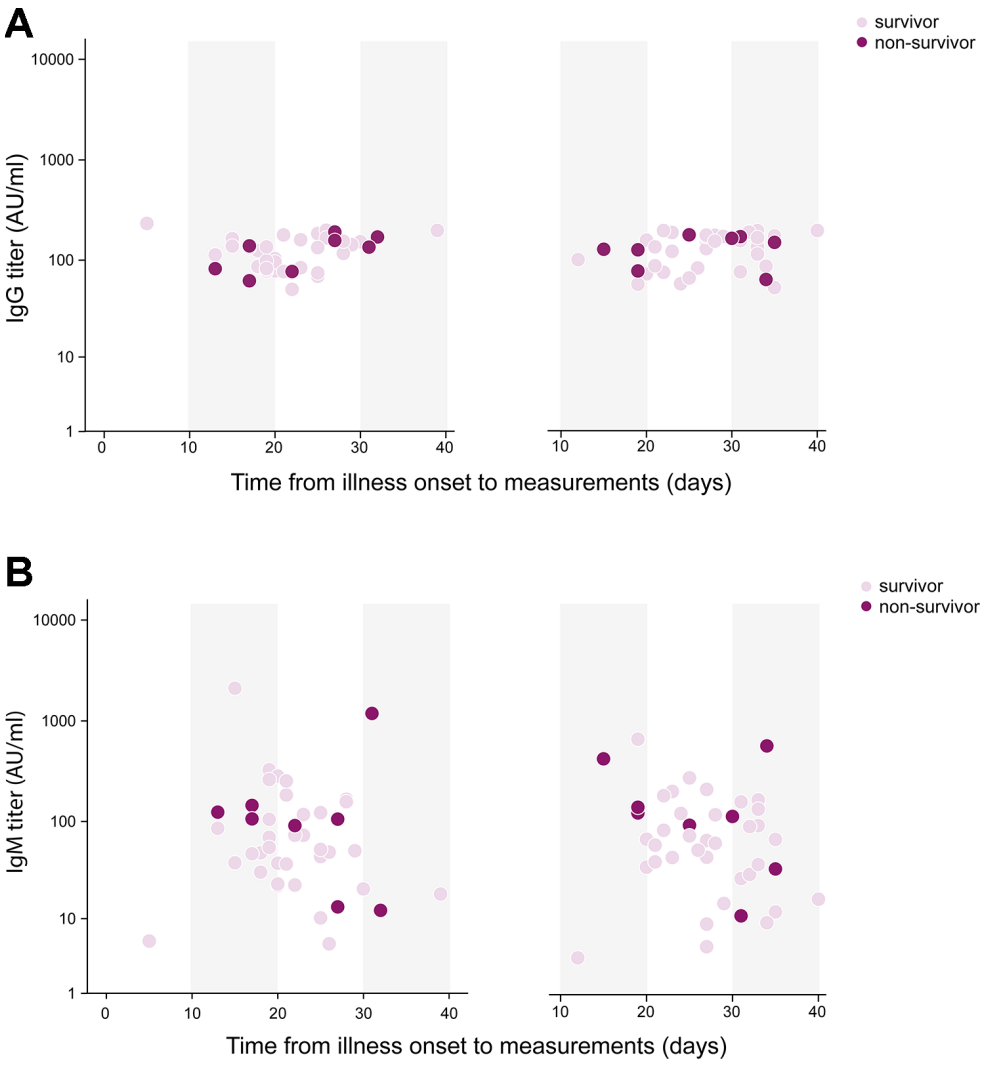

Figure 2. Temporal profile of serum antibodies in severe/critical patients with COVID-19. 42 patients had two antibody measurements on day 25 (SD, 7) and on day 27 (SD, 6) post illness onset respectively. (**A**) IgG titer remained stable during two measurements in both survivors and non-survivors. (**B**) Change of IgM titer in survivors showed a significantly decreasing (-4 [IQR -14-0], P=0.031), but that in non-survivors didn't show statistical difference (3 [IQR -19-29], P=0.779).

Table 1. Clinical characteristics of patients with different IgM titers.

	IgM < 50 AU/ml (n=39)	IgM ≥ 50 AU/ml (n=40)	*P*
Age, years	64±11	61±14	0.315
Men	25(64)	25(63)	0.883
Current smoker	5(13)	2(5)	0.221
Comorbidity			
Chronic obstructive lung disease	3(8)	2(5)	0.623
Hypertension	17(44)	14(35)	0.434
Diabetes	7(18)	6(15)	0.724
Coronary heart disease	4(10)	2(5)	0.378
Chronic kidney disease	0(0)	2(5)	0.494
Symptoms			
Fever	30(77)	34(85)	0.360
Cough	28(72)	29(73)	0.944
Sputum	15(38)	11(28)	0.300
Myalgia	1(3)	5(13)	0.201
Fatigue	22(56)	22(55)	0.900
Diarrhoea	6(15)	6(15)	0.962
Dyspnea	25(64)	24(60)	0.707
Time from illness onset to hospital admission, days	10(7-14)	12(10-14)	0.172
Time from illness onset to first antibody detection, days	26(21-31)	23(19-29)	0.183
Time from hospital admission to first antibody detection, days	13(9-21)	11(7-15)	0.153
Vital signs on admission			
Temperature, °C	36.9±0.6	36.9±0.9	0.774
Systolic pressure, mmHg	129±18	128±18	0.857
Diastolic pressure, mmHg	78±12	76±9	0.461
Heart rate, beats/min	91±18	87±14	0.275
Disease severity state			0.003
Severe	36(92)	26(65)	
Critical	3(8)	14(35)	

Data are mean ± SD, median (IQR) or n (%). IgM = Immunoglobulin M.

Table 2. Laboratory measurements of patients with different IgM titers.

	IgM < 50 AU/ml (n=39)	IgM ≥ 50 AU/ml (n=40)	*P*
Arterial blood gas analysis			
PH	7.38±0.06	7.40±0.05	0.136
PaCO2, mmHg	44±7	42±8	0.277
PaO2, mmHg	59±6	56±7	0.044
SaO2, %	91±4	89±4	0.039
White blood cell count, $\times 10^9$/L	6.9±3.0	7.1±2.8	0.777
Neutrophil count, $\times 10^9$/L	5.5±2.9	5.8±2.9	0.608
Lymphocyte count, $\times 10^9$/L	0.9±0.4	0.9±1.0	0.800
Haemoglobin, g/L	126±15	126±19	0.812
Platelet count, $\times 10^9$/L	249±118	228±87	0.369
ALT, U/L	24(18-44)	39(16-63)	0.161
Albumin, g/L	34±4	32±5	0.010
Creatinine, μmol/L	86±26	83±38	0.730
Prothrombin time, s	12.0±0.8	12.4±1.2	0.085

Fibrinogen, g/L	5.0±1.9	5.2±1.7	0.623
D-dimer, mg/L	0.95(0.44-2.59)	1.81(0.77-9.06)	0.020
Cardiac troponin T, pg/ml	10(6-18)	12(8-20)	0.666
NT-proBNP, pg/ml	80(59-252)	264(73-590)	0.031
C-reactive protein, mg/L	40(12-107)	69(27-126)	0.119
IL-6, pg/mL	17(6-70)	42(12-119)	0.141
TNF-α, pg/mL	11(8-17)	9(5-12)	0.111

Data are mean ± SD or median (IQR). IgM = Immunoglobulin M. PH = Pondus Hydrogenii. PaCO2 = partial pressure of carbon dioxide. PaO2 = partial pressure of oxygen. SaO2 = arterial oxygen saturation. ALT = alanine aminotransferase. NT-proBNP = N-terminal pro-brain natriuretic peptide. IL-6=interleukin-6. TNF-α = tumor necrosis factor-α.

Table 3. Treatments and outcomes of patients with different IgM titers

	IgM < 50 AU/ml (n=39)	**IgM ≥ 50 AU/ml (n=40)**	***P***
Drugs			
Antiviral treatment	36(92)	38(95)	0.675
Antibiotics	36(92)	39(98)	0.359
Corticosteroids	16(41)	32(80)	<0.001
Chinese traditional medicine	39(100)	39(98)	1.000
Oxygen inhalation	38(97)	38(95)	0.571
Mechanical ventilation	3(8)	14(35)	0.003
Non-invasive	3(8)	13(33)	0.006
Invasive	0(0)	9(23)	0.002
Other advanced supportive therapy	1(3)	4(10)	0.175
IABP	0(0)	1(3)	0.320
CRRT	1(3)	4(10)	0.175
ECMO	0(0)	2(5)	0.157
Outcomes			
ARDS	2(5)	14(35)	0.001
Septic shock	2(5)	9(23)	0.026
In-hospital mortality	2(5)	9(23)	0.026
Hospital length of stay, days	29(21-30)	29(19-31)	0.941

Data are median (IQR) or n (%). IgM = Immunoglobulin M. IABP = intra-aortic balloon pump. CRRT = continuous renal replacement therapy. ECMO = extracorporeal membrane oxygenation. ARDS = Acute Respiratory Distress Syndrome.

Serum IgM is the first protein producing in human in response to the exposure to an antigen, such as bacterial, virus, and others. IgM titer could increase in hours to respond antigen attack followed by degradation in weeks. Being a secondly important antibody, IgG would be activated in a moderate but long-lasting way. It might slowly rise in weeks after recognizing antigen, and reach a plateau for years. Guo et al. examined 208 samples from confirmed and suspected patients with COVID-19. Specific antibodies could be positive as early as day 1 after illness onset. For most patients, IgM appeared at day 5 and became stable at days 15-21 after increasing at day 8. IgG showed same change as IgM at acute phase but continued its rising until plateau at day 21 [13]. Our patients had their antibody measurement on day 25 (SD7), and repeated on day 27 (SD6). Despite of the stable levels in IgG and IgM, our measurements were performed later than other studies. We believed the results were still robust because the measurements were performed at the time when both IgG and IgM were in plateau according to previous studies [21], and the IgG and IgM titers remained high and detectable in our study. Moreover, we observed IgM might decrease on day 27 (SD 6) if patients recovered. As Mo et al mentioned in their study, IgM against SARS-CoV declined much earlier than IgG [22]. The decreasing of IgM against SARS-CoV-2 in survivors from our study might be a natural change of IgM in COVID-19. On the other hand, To et al. investigated the correlation between serum antibody response and viral load. They found IgG and IgM titers were highly correlated with viral load in patients with COVID-19, which might explain why our patients had a recover in their illness in consistent with IgM decreasing [14]. One thing might be noticed, there were 10 patients having negative molecular tests in our study, even though they presented critical illness. Similar findings were seen in the

study by Zhang et al. They observed positive rate in molecular tests might be reducing as time from illness onset prolonged, while IgG and IgM titers were stable in all patients [23]. The reasons for this were discussed before: viral RNA might vary from oral swabs to anal swabs; mismatch in the detection probes; fluctuation in viral load unparalleled with illness progression [24, 25].

Efforts have been made to distinguish patients at high risk of mortality. Studies proposed age, comorbidities, CT imaging, and other parameters, which showed differences in survivors and non-survivors [26, 27], to predict risks in patients with COVID-19. Nonetheless, we didn't find many differences between survivors and non-survivors in our study. Severe and critical illness in our patients might eliminate the influence by other factors. On the contrary, our study supported the clinical application of serum IgM in severe/critical patients with COVID-19 for risk stratification. Significantly higher mortality rate was seen in patients when their serum IgM was higher than 50 AU/ml. Additionally, serial changes in IgM titer also helped to follow the disease progression in patients with poor prognosis.

Our study showed that advanced supportive treatment together with combination therapy were more applied in patients with high mortality. The high levels of IgM in our patients might indicate a disease worsening despite of the treatment. Treatment strategy was proposed based on the disease stage, however, no evidence had been shown to be most specific to COVID-19 [28]. Patients might show different response to corticosteroids [29, 30]. Although patients admitted into ICU required more medical treatments, the effect of advanced support seemed to be controversial in critical patients [31, 32]. The ideally strategy to treat viral pneumonia has always been remove the virus as soon as possible. The antivirus effect by Remdesivir in patient and cells might bring hope in further treatment [33, 34].

There were some limitations in our studies. Firstly, there were only 79 patients included in our study. The small size of study population might bring bias to data distribution. Further study should involve more patients to investigate the clinical profile of antibody response. Secondly, our antibody measurements started on 25 days post illness onset. The late measurements missed early change of antibody in patients. New studies might consider a broader interval to cover more changes. Thirdly, we focused on in-hospital mortality for severe/critical patients. However, there were reports that patients might have disease progression after discharge [24]. We might follow-up our patients for a longer time to see the relationship between antibody titer and their prognosis.

CONCLUSIONS

Our study demonstrated the dynamic change of antibody titer in consistent with disease progression. A higher risk of in-hospital mortality was seen in severe/critical patients of COVID-19 when their IgM titer ≥ 50 AU/ml. Further decreasing of IgM could imply a better prognosis in severe/critical patients. Serial measurements of serum antibody provide comprehensive evaluation to the process of COVID-19.

MATERIALS AND METHODS

Study design and patients

A retrospective cohort study was conducted in Wuhan Asia General Hospital, Wuhan, China to investigate the clinical profile of serum antibodies against SARS-CoV-2 in severe/critical patients with COVID-19. The study protocol was reviewed and approved by the Ethics Committee of Wuhan Asia General hospital with a waiver of informed consent (WAGHMEC-KY-2020007). Personal information of patients was re-identified before analysis.

A total of 105 severe/critical patients with COVID-19 admitted in Wuhan Asia General hospital between 2020.01.22 and 2020.03.06 were reviewed. COVID-19 was diagnosed according to the Chinese management guideline for COVID-19 (version 7.0) [6]. New laboratory criteria of COVID-19-specific IgM and IgG positive, and 4 folds increasing of COVID-19-specific IgG titer in recovery period were added in guideline 7.0 [6]. Severe patients with COVID-19 met any of the followings: (1) Shortness of breath, respiratory rate ≥ 30 times per minute; (2) Oxygen saturation ≤ 93% at rest; (3) Alveolar oxygen partial pressure/fraction of inspiration O2 (PaO2/FiO2) ≤ 300 mmHg (1mmHg=0.133kPa). Critical patients had any of the conditions: (1) Respiratory failure requiring mechanical ventilation; (2) Shock; (3) Patients combined with other organ failure needed intensive care unit (ICU) monitoring and treatment [6]. Fever was defined as axillary temperature greater than 37·3°C.

Data collection

Clinical data including age, gender, vital signs, comorbidity were collected from medical records at admission. Laboratory biomarkers such as IgG titer, IgM titer, blood gas analysis, white blood cell count (WBC), alanine aminotransferase (ALT), D-dimer, and N-terminal prohormone of brain natriuretic peptide (NT-proBNP) were also collected. Specifically, serum IgG and IgM that against SARS-CoV-2 nucleocapsid protein and envelop protein were measured by

chemiluminescence immunoassay (CLIA) in automatic system when it was available on February 18, 2020. Antibody titer > 10 AU/ml was taken as positive. All blood tests were analyzed in fresh blood and determined by standard quantitative assay techniques in our Department of Clinical Laboratory according to the manufacturer's protocol.

Outcomes

The primary outcome was in-hospital mortality. The secondary outcomes included ARDS related to SARS-CoV-2 infection and sepsis shock secondary to COVID-19. ARDS was diagnosed according to the Berlin Definition [35]. Sepsis shock was defined according to the 2016 Third International Consensus Definition [36].

Statistical analysis

Data are shown as number for categorical data, and mean ± standard deviation or median with interquartile range (IQR) as appropriate for continuous data. Data were compared with student t test or Wilcoxon signed-rank test for continuous variables depending on the normality of their distributions and with the $\chi 2$ test for categorical variables. Comparison between the first and second antibody titer is performed by paired samples Wilcoxon test. A two-side $P < 0.05$ was considered as statistic significant. All statistical analyses were performed with SPSS 23.0 (IBM Corp, Armonk, NY).

Abbreviations

ALT: alanine aminotransferase; ARDS: acute respiratory distress syndrome; CAD: coronary artery disease; CKD: chronic kidney disease; COPD: chronic obstructive pulmonary disease; COVID-19: coronavirus disease 2019; CLIA: chemiluminescence immunoassay; ICU: intensive care unit; IgG: immunoglobulin G; IQR: interquartile range; MERS-CoV: Middle East Respiratory Syndrome-coronavirus; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; PaO2/FiO2: Alveolar oxygen partial pressure/fraction of inspiration O2; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; SD: standard deviation; WBC: white blood cell count.

AUTHOR CONTRIBUTIONS

Xintian Liu and Xuan Zheng designed the study. Bo Liu and Mingxiang Wu collected the epidemiological and clinical data. Zhenlu Zhang provided laboratory measurements and collected the data. Xintian Liu, Xuan Zheng and Xi Su summarized all data. Gangcheng Zhang, Xi Su, Xintian Liu and Xuan Zheng drafted the manuscript. All the authors proved the final version of this manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

3. Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A, Al-Jabir A, Iosifidis C, Agha R. World health organization declares global emergency: a review of the 2019 novel coronavirus (COVID-19). Int J Surg. 2020; 76:71–76. https://doi.org/10.1016/j.ijsu.2020.02.034 PMID:32112977

4. Cucinotta D, Vanelli M. WHO declares COVID-19 a pandemic. Acta Biomed. 2020; 91:157–60. https://doi.org/10.23750/abm.v91i1.9397 PMID:32191675

5. Li T. Diagnosis and clinical management of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: an operational recommendation of peking

union medical college hospital (V2.0). Emerg Microbes Infect. 2020; 9:582–85.
https://doi.org/10.1080/22221751.2020.1735265
PMID:32172669

6. Commission CNH. Chinese Clinical Guidance for COVID-19 Pneumonia Diagnosis and Treatment (7th edition). 2020.

7. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81.
https://doi.org/10.1016/S2213-2600(20)30079-5
PMID:32105632

8. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. JAMA. 2020. [Epub ahead of print].
https://doi.org/10.1001/jama.2020.2648
PMID:32091533

9. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62.
https://doi.org/10.1016/S0140-6736(20)30566-3
PMID:32171076

10. Li K, Wu J, Wu F, Guo D, Chen L, Fang Z, Li C. The clinical and chest CT features associated with severe and critical COVID-19 pneumonia. Invest Radiol. 2020; 55:327–31.
https://doi.org/10.1097/RLI.0000000000000672
PMID:32118615

11. Mo HY, Xu J, Ren XL, Zeng GQ, Tan YX, Chen RC, Chan-Yeung M, Zhong NS. Evaluation by indirect immunofluorescent assay and enzyme linked immunosorbent assay of the dynamic changes of serum antibody responses against severe acute respiratory syndrome coronavirus. Chin Med J (Engl). 2005; 118:446–50.
PMID:15788124

12. Wang WL, Wang HJ, Deng Y, Song T, Lan JM, Wu GZ, Ke CW, Tan WJ. Serological study of an imported case of middle east respiratory syndrome and his close contacts in China, 2015. Biomed Environ Sci. 2016; 29:219–23.
https://doi.org/10.3967/bes2016.027
PMID:27109133

13. Guo L, Ren L, Yang S, Xiao M, Chang D, Yang F, Dela Cruz CS, Wang Y, Wu C, Xiao Y, Zhang L, Han L, Dang S, et al. Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19). Clin Infect Dis. 2020. [Epub ahead of print].
https://doi.org/10.1093/cid/ciaa310
PMID:32198501

14. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, Yip CC, Cai JP, Chan JM, Chik TS, Lau DP, Choi CY, Chen LL, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis. 2020; 20:565–74.
https://doi.org/10.1016/S1473-3099(20)30196-1
PMID:32213337

15. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579:270–73.
https://doi.org/10.1038/s41586-020-2012-7
PMID:32015507

16. Porcheddu R, Serra C, Kelvin D, Kelvin N, Rubino S. Similarity in case fatality rates (CFR) of COVID-19/SARS-COV-2 in Italy and China. J Infect Dev Ctries. 2020; 14:125–28.
https://doi.org/10.3855/jidc.12600
PMID:32146445

17. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, Li SB, Wang HY, Zhang S, Gao HN, Sheng JF, Cai HL, Qiu YQ, Li LJ. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-cov-2) outside of wuhan, China: retrospective case series. BMJ. 2020; 368:m606.
https://doi.org/10.1136/bmj.m606 PMID:32075786

18. Young BE, Ong SW, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, et al, and Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. JAMA. 2020; 323:1488–94.
https://doi.org/10.1001/jama.2020.3204
PMID:32125362

19. Ge H, Wang X, Yuan X, Xiao G, Wang C, Deng T, Yuan Q, Xiao X. The epidemiology and clinical information about COVID-19. Eur J Clin Microbiol Infect Dis. 2020; 39:1011–1019.
https://doi.org/10.1007/s10096-020-03874-z
PMID:32291542

20. Bhatraju PK, Ghassemieh BJ, Nichols M, Kim R, Jerome KR, Nalla AK, Greninger AL, Pipavath S, Wurfel MM, Evans L, Kritek PA, West TE, Luks A, et al. Covid-19 in critically ill patients in the seattle region - case series. N Engl J Med. 2020; 382:2012–22.

https://doi.org/10.1056/NEJMoa2004500
PMID:32227758

21. Xiao DAT, Gao DC, Zhang DS. Profile of Specific Antibodies to SARS-CoV-2: The First Report. J Infect. 2020; 81:147-78.
https://doi.org/10.1016/j.jinf.2020.03.012

22. Mo H, Zeng G, Ren X, Li H, Ke C, Tan Y, Cai C, Lai K, Chen R, Chan-Yeung M, Zhong N. Longitudinal profile of antibodies against SARS-coronavirus in SARS patients and their clinical significance. Respirology. 2006; 11:49–53.
https://doi.org/10.1111/j.1440-1843.2006.00783.x
PMID:16423201

23. Zhang W, Du RH, Li B, Zheng XS, Yang XL, Hu B, Wang YY, Xiao GF, Yan B, Shi ZL, Zhou P. Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes. Emerg Microbes Infect. 2020; 9:386–89.
https://doi.org/10.1080/22221751.2020.1729071
PMID:32065057

24. Lan L, Xu D, Ye G, Xia C, Wang S, Li Y, Xu H. Positive RT-PCR test results in patients recovered from COVID-19. JAMA. 2020; 323:1502–03.
https://doi.org/10.1001/jama.2020.2783
PMID:32105304

25. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020; 25:2000045.
https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045
PMID:31992387

26. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Medicine. 2020; 46:1294-97.
https://doi.org/10.1007/s00134-020-06028-z

27. Jung SM, Akhmetzhanov AR, Hayashi K, Linton NM, Yang Y, Yuan B, Kobayashi T, Kinoshita R, Nishiura H. Real-time estimation of the risk of death from novel coronavirus (COVID-19) infection: inference using exported cases. J Clin Med. 2020; 9:523.
https://doi.org/10.3390/jcm9020523
PMID:32075152

28. Li G, De Clercq E. Therapeutic options for the 2019 novel coronavirus (2019-nCoV). Nat Rev Drug Discov. 2020; 19:149–50.
https://doi.org/10.1038/d41573-020-00016-0
PMID:32127666

29. Shang L, Zhao J, Hu Y, Du R, Cao B. On the use of corticosteroids for 2019-nCoV pneumonia. Lancet. 2020; 395:683–84.
https://doi.org/10.1016/S0140-6736(20)30361-5
PMID:32122468

30. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. Lancet. 2020; 395:473–75.
https://doi.org/10.1016/S0140-6736(20)30317-2
PMID:32043983

31. Arabi YM, Murthy S, Webb S. COVID-19: a novel coronavirus and a novel challenge for critical care. Intensive Care Med. 2020; 46:833–36.
https://doi.org/10.1007/s00134-020-05955-1
PMID:32125458

32. Ñamendys-Silva SA. Respiratory support for patients with COVID-19 infection. Lancet Respir Med. 2020; 8:e18.
https://doi.org/10.1016/S2213-2600(20)30110-7
PMID:32145829

33. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 2020; 30:269–71.
https://doi.org/10.1038/s41422-020-0282-0
PMID:32020029

34. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, Spitters C, Ericson K, Wilkerson S, Tural A, Diaz G, Cohn A, Fox L, et al, and Washington State 2019-nCoV Case Investigation Team. First case of 2019 novel coronavirus in the United States. N Engl J Med. 2020; 382:929–36.
https://doi.org/10.1056/NEJMoa2001191
PMID:32004427

35. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS, and ARDS Definition Task Force. Acute respiratory distress syndrome: the berlin definition. JAMA. 2012; 307:2526–33.
https://doi.org/10.1001/jama.2012.5669
PMID:22797452

36. Shankar-Hari M, Phillips GS, Levy ML, Seymour CW, Liu VX, Deutschman CS, Angus DC, Rubenfeld GD, Singer M, and Sepsis Definitions Task Force. Developing a new definition and assessing new clinical criteria for septic shock: for the third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA. 2016; 315:775–87.
https://doi.org/10.1001/jama.2016.0289
PMID:26903336

Research Paper

Questionnaire assessment helps the self-management of patients with inflammatory bowel disease during the outbreak of Coronavirus Disease 2019

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ABSTRACT

Objective: This study aimed to assess the disease conditions of patients with inflammatory bowel disease (IBD) in Hubei Province during the outbreak of Coronavirus Disease 2019 (COVID-19) by questionnaire online and guide their self-management during this epidemic.

Results: A total of 102 eligible questionnaires were included. No patient we surveyed reported a diagnosis of COVID-19. Our result showed that 67.86% of patients with ulcerative colitis (UC) and 80.43% of patients with Crohn's disease (CD) were in remission, 85.29%of patients had a good quality of life. Part of the patients (21.57%) reported their disease conditions worsening. The reduction in physical exercise was a risk factor for worsening conditions (OR=17.593, p=0.009). Some patients reported an alteration of medication regimens during the epidemic.

Conclusions: The epidemic of COVID-19 might have a certain impact on many aspects of Hubei IBD patients within four weeks after the traffic control. Doctors could utilize the results from our questionnaire to guide IBD patients' self-management.

Methods: A questionnaire was designed containing the Harvey-Bradshaw Index (HBI), the 6-point Mayo Score, the short inflammatory bowel disease questionnaire (SIBDQ) and distributed to Hubei IBD patients online within four weeks of traffic control after the outbreak, it also included questions about patients' self-reported disease conditions and their epidemiological features of COVID-19.

INTRODUCTION

In December 2019, an outbreak of COVID-19 caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, the capital of Hubei Province, China [1, 2]. On January 23, 2020, the Chinese government implemented traffic controls to prevent the spread of COVID-19 [3]. We distributed questionnaires to IBD patients from February 18th to 20th, which is approximately four weeks after traffic control. As of 20 February 2020, there had been 75,465 cases of COVID-19 confirmed in mainland China, including 62,662 cases in Hubei Province. The number of confirmed diagnoses in Hubei Province is 83.03% of that in China, accounting for the majority. At the same time, medical resources had also shifted more towards

the diagnosis and treatment of COVID-19 in Hubei Province. These factors made routine medical treatment and follow-up of patients with chronic diseases inconvenient. Under these influences, it is essential for patients with chronic diseases to self-manage under the guidance of the doctor in this particular period. Self-management is the process by which patients participate in decision-making and self-care under the guidance of the doctors [4]. Patient's effective self-management can relieve symptoms and control disease activity to a certain extent [5].

Inflammatory bowel disease (IBD) is a type of chronic idiopathic bowel disease, includes Crohn's disease (CD) and ulcerative colitis (UC). IBD patients have varying degrees of immune disorders [6] so that they may be considered as virus-susceptible. It is particularly crucial for IBD patients to know how to manage by themselves during the epidemic. Self-management requires monitoring of diseases by doctors first [7]. We monitored the patient's disease status through a questionnaire in our study. The content of the survey and the concept of the questionnaire design came from the patient-reported outcome (PRO). PRO is a visual report of the patient's treatment and disease management results, emphasizing the patient's self-evaluation and subjective perception [8]. PRO contains objective and subjective evaluation contents, which may include disease status, changes in functional status before and after the intervention, HRQoL and the patient's personal impressions [9–12].

To assess patients' disease activity, HRQoL, and self-reported disease conditions, we design a verified 60-item questionnaire based on the concept of PRO. First of all, our questionnaire included the 6-point Mayo, HBI and SIBDQ. These indexes were objective quantitative indicators designed to obtain detailed knowledge of the disease activity and HRQoL of these IBD patients. Secondly, there were questions to understand patients' subjective perceptions of disease conditions. Finally, this questionnaire also included questions about COVID-19 epidemiological features of these IBD patients. We gave feedback to these IBD patients and guided them to develop targeted self-management programs after obtaining the information through the questionnaire.

RESULTS

Demographic characteristics of study participants

A proximately 350 electronic questionnaires were distributed and a total of 111 were returned. There were 102 valid questionnaires, with an effective rate of 91.89%. The nine questionnaires excluded were due to some missing items. The median age of participants was 34 years (IQR, 27.25-42.25; range, 14-66), and 66.67% of participants were men. There were 56 (54.90%) patients with ulcerative colitis and 46 (45.10%) patients with Crohn's disease. Among all the participants in our survey, no one has reported infection with SARS-CoV-2; no one had symptoms related to COVID-19 or had a history of exposure. Table 1 shows the demographic data and disease-related variables for all participants who agreed and completed the survey.

Disease activity

We used the 6-point Mayo and HBI to score and grade disease activity in UC and CD patients respectively. The results were shown in Table 2. The median 6-point Mayo score of UC patients was 1 (IQR, 0-3; range, 0-6). Of the 56 UC patients, 38 (67.86%) UC patients were in remission, 4 (7.14%) patients had mild activity, 13 (23.21%) patients had moderate activity, and 1 (1.79%) patients had severe activity. The median HBI of CD patients was 2 (IQR, 1-4; range, 0-12). Of the 46 CD patients, 37 (80.43%) CD patients were in remission, 4 (8.70%) patients had mild activity, 5 (40.87%) patients had moderate activity, and no patients had severe activity. There was not a statistically significant difference in the proportion of the disease activity stage between UC and CD patients ($p = 0.301$).

Quality of life

The median SIBDQ of all participants was 59 (IQR, 52.25-63; range, 34-70). The median SIBDQ of UC patients was 60 (IQR, 54.75-64; range, 35-70), and the median SIBDQ of CD patients was 58 (IQR, 52-62.75; range, 34-69). Among all participants, 87 (85.29%) patients had the good health-related quality of life (HRQoL) (SIBDQ $\geq$ 50). There were 49 (87.50%) UC patients who had good HRQoL, compared with 38 (82.61%) CD patients who had good HRQoL ($p=0.338$) (Table 3), suggesting HRQoL is not significantly different between UC and CD patients.

Self-reported disease conditions

In this questionnaire, we investigated the change of the patient's self-reported disease condition through the patient's subjective report. Approximately half of the patients (n = 55, 53.92%) thought that their disease condition did not change during the epidemic, 25 (24.51%) considered their disease condition improved, and 22 (21.57%) considered their disease condition worsening. We attempted to study the risk factors of change in patients' self-reported disease conditions. The

Table 1. Baseline characteristics of the study population. (n=102).

Characteristics	Value
Age, Median (min-max) (IQR), y	34 (14-66) (27.25-42.25)
Gender	
Female	34 (33.33%)
Male	68 (66.67%)
Diagnosis	
Ulcerative Colitis	56 (54.90%)
Crohn's Disease	46 (45.10%)
Diagnosed with COVID-19	0
Huanan seafood market exposure	0
Signs and symptoms of COVID-19	0
Habitation*	
Wuhan	26 (25.49%)
Xiaogan	22(21.57%)
Jingzhou	12 (11.76%)
Suizhou	8 (7.83%)
Xiangyang	6 (5.88%)
Huangshi	6 (5.88%)
Huanggang	5 (4.90%)
Yichang	5 (4.90%)
Jingmen	4 (3.92%)
Xianning	2 (1.96%)
Xiantao	2 (1.96%)
Enshi Tujia and Miao Autonomous Prefecture	2(1.96%)
Tianmen	1 (0.98%)
Qianjiang	1 (0.98%)

*All participants are located in Hubei Province.

Table 2. Evaluation of participants' IBD disease activity.

	Participants with Ulcerative Colitis(n=56)	Participants with Crohn's Disease(n=46)	P-value*
Index, Median (IQR) (range)	6-point Mayo,1(0-3)(0-6)	HBI, 2 (1-4) (0-12)	
Disease activity stage			0.301
Remission phase, n (%)	38 (67.86%)	37 (80.43%)	
Mild active phase, n (%)	4 (7.14%)	4 (8.70%)	
Moderate active phase, n (%)	13 (23.21%)	5 (10.87%)	
Severe active phase, n (%)	1 (1.79%)	0 (0%)	

HBI: Harvey-Bradshaw Index.
*Chi-square test was used to test whether there was a statistically significant difference in the proportion of the disease activity between UC and CD patients.

Table 3. Evaluation of participants' SIBDQ.

	Total participants (n=102)	Participants with UC and CD respectively		
		Participants with UC (n=56)	Participants with CD (n=46)	P-value*
SIBDQ, Median (IQR)	59 (52.25-63)	60 (54.75-64)	58 (52-62.75)	
HRQoL				0.338
Good (≥ 50[1]), n (%)	87 (85.29%)	49 (87.50%)	38 (82.61%)	
Poor (<50), n (%)	15 (14.71%)	7 (15.50%)	8 (17.39%)	

HRQoL: health-related quality of life
1: SIBDQ score of more than 50 are considered to have a good HRQoL
*Chi-square test was used to test whether there was a statistically significant difference in the proportion of HRQoL between UC and CD patients.

result showed that reduced physical exercise was a risk factor for worse in the disease condition (OR=17.593, 95%CI 2.035 to 152.097, p=0.009). The other factors did not have a significant risk for change in the patient's disease condition. These data are shown in Table 4. The status of these factors during the epidemic came from the patients' personal reports.

The change in medication regimen

We studied participants' medication regimens before and after the outbreak of COVID-19. Among UC patients, there was an increase of 3 patients who took no medication due to the discontinuation of mesalazine. The reason for the withdrawal of mesalazine was that the medicine was not available. Among CD patients, the number of patients who used adalimumab or took no medication increased, and the number of patients who used Remicade or methotrexate decreased. The details were shown in Table 5. The reasons for changing the medication regimens of these CD patients were "inability to purchase medication" and "inability to go to the hospital for routine treatment". Among the reasons for these patients who changed their medication regimens, no one chose the options of "forgetting to take medicine" and "reducing medicine on your own". This result represented that IBD patients' medical compliance during the epidemic was excellent in our survey.

Emotional state

Negative emotions are significantly correlated with clinical recurrence and are also considered to be independent risk factors for more frequent relapse of disease [13, 14]. Therefore, we also investigated the emotional states of IBD patients in this survey. Changes in emotional states came from the patients' self-judgments. More than half of the participants (57.85%) thought they had the ordinary moods during this epidemic, 35.29% had positive moods, and 6.86% had negative moods.

DISCUSSION

The COVID-19 epidemic outbreak emerged in Wuhan, Hubei Province of China in December 2019 [15]. The population was generally susceptible to SARS-CoV-2, especially for the elderly and the people with underlying diseases, who are prone to serious consequences [16, 17]. In our survey, there was no patient reported infection with SARS-CoV-2, but this did not mean that IBD patients were not susceptible to SARS-CoV-2 since our small sample size and the participants of this study were not obtained by randomized sampling. Until now, there is no evidence to prove the susceptibility of IBD patients to COVID-19 from other studies [18]. The epidemic led to the difficulty of maintaining previous disease management for IBD patients due to the contraction in routine medical resources. Our research focused on guiding the patient's self-management through the results of the questionnaire.

The traffic control in China from late January might largely change the lifestyle, psychological, and physical condition of the Chinese population [19–21], especially for residents in Hubei Province. We used the web questionnaire to evaluate the disease activity, HRQoL and the self-reported disease condition of IBD patients. Then we provided feedback on the patients' conditions and advised on their self-management. This manner helped IBD patients to adjust their treatment plans and develop a home-based self-management medical intervention model. Active doctor-patient communication can improve patients' confidence with treatment, shared decision-making capacity and then provide a good impact on disease activity [4, 22]. We will continue to distribute questionnaires to this group of IBD patients in Hubei Province every month to guide patients' home-based self-management during the epidemic of COVID-19.

Our results showed that 67.86% of UC patients and 80.43% of CD patients were in remission assessed by the 6-point Mayo score and HBI index. 85.29% of patients had a good HRQoL during the epidemic through the SIBDQ test. This suggested that more than half of the patients were in remission and had a good HRQoL in the early period of traffic control after the outbreak of COVID-19. With regard to the patients' self-reported results, although 78.43% of the patients thought that their disease conditions had not changed or even improved, there were still 21.57% of the patients considered that their disease conditions worsening. This showed that the early epidemic also had a certain impact on the patient's disease condition.

We studied the influencing factors of the self-reported disease condition of IBD patients during this epidemic. The factors included "reduction of exercise", "emotional state", "change of medication regimen", "daily rest" and "subsequent visit". The results showed that the reduction of exercise was a risk factor for worsening disease (OR=17.593, p=0.009). The other factors that could affect the disease conditions of IBD patients in previous studies [23–27] didn't show a statistical correlation in our survey. This might be because of our small sample size. Therefore, we still recommend that IBD patients maintain a positive emotional state, retain the medication regimen, have adequate rest and make timely doctor-patient communication during the self-management process.

Table 4. Multi-variable logistic regression of the causes of change in participants' self-evaluation disease condition.

Disease condition	Characters	n (%)	OR(95%CI)	P-value
Condition worsening (n=22)		Subsequent visit		
	No	18 (81.82%)	3.785(0.871, 16.458)	0.076
	Yes	4 (18.18%)		
		Medication regimen		
	Not changed	14 (63.64%)	0.264 (0.060, 1.167)	0.079
	Changed	8 (36.36%)		
		Emotional status		
	Negative	4 (18.18%)	3.306(0.413, 26.454)	0.260
	Positive	3 (13.64%)	0.830 (0.178, 3.880)	0.813
	Normal	15 (68.18%)		
		Physical exercise		
	Reduced	5 (22.73%)	17.593 (2.035, 152.097)	0.009
	Not reduced	17 (77.27%)		
		Rest		
	Inadequate	21 (95.45%)	1.071 (0.262, 4.378)	0.924
	Adequate	1 (4.55%)		
		Smoking		
	No	19 (86.36%)	0.665 (0.123, 3.591)	0.636
	Yes	3 (13.64%)		
Condition improved (n=25)		Subsequent visit		
	No	21 (84.00%)	2.730 (0.731, 10.199)	0.135
	Yes	4 (16.00%)		
		Medication regimen		
	Not changed	19 (76.00%)	0.374 (0.094, 1.496)	0.165
	Changed	6 (24.00%)		
		Emotional status		
	Negative	1 (4.00%)	2.094 (0.138, 31.710)	0.594
	Positive	14 (56.00%)	2.259 (0.751, 6.792)	0.147
	Normal	10 (40.00%)		
		Physical exercise		
	Reduced	1 (4.00%)	0.694 (0.222, 2.167)	0.529
	Not reduced	24 (96.00%)		
		Rest		
	Inadequate	9 (36.00%)	0.136 (0.015, 1.249)	0.078
	Adequate	16 (64.00%)		
		Smoking		
	Yes	4 (15.38%)	0.481 (0.104, 2.228)	0.350
	No	22 (84.62%)		

*The category with no change in the condition (n=55) was used as the reference category for the two groups of condition worsening (n=22) and condition improved (n=25).

Pharmacological intervention is a key part of managing symptoms and maintaining remission in patients with IBD [28]. Our results showed that the number of people who took no medication increased among UC and CD patients after the outbreak of COVID-19. The number of UC patients taking mesalazine decreased due to the inconvenience of obtaining medications. For CD patients, the use of Remicade or methotrexate decreased because Remicade infusion in the hospital was not accessible and methotrexate could not be purchased.

Table 5. The comparison of the medication regimens before and after the outbreak of COVID-19 and the changes in medication regimens.

	Medication	Before the outbreak, n (%)	After the outbreak, n (%)	The number of changes*, n
UC patients, (n=56)	No medication	1 (1.75%)	4 (5.26%)	+3
	Mesalazine	50 (89.47%)	47 (85.96%)	-3
	Enteral nutrition	1 (1.75%)	1 (1.75%)	0
	Glucocorticoid	2 (3.51%)	2 (3.51%)	0
	Probiotics	4 (5.26%)	4 (5.26%)	0
	Biological therapy			
	Remicade	3 (5.26%)	3 (5.26%)	0
	Adalimumab	1 (1.75%)	1 (1.75%)	0
CD patients, (n=46)	No medication	1 (1.92%)	3 (5.77%)	+2
	Mesalazine	8 (17.31%)	8 (17.31%)	0
	Enteral nutrition	10 (19.23%)	10 (21.15%)	0
	Glucocorticoid	2 (3.85%)	2 (3.85%)	0
	Probiotics	1 (1.92%)	1 (1.92%)	0
	Biological therapy			
	Remicade	21 (46.15%)	16 (36.54%)	-5
	Adalimumab	1 (1.92%)	2 (3.85%)	+1
	Immunomodulators			
	Methotrexate	2 (3.85%)	1 (1.92%)	-1
	Azathioprine	20 (40.38%)	20 (40.38%)	0
	Thalidomide	1 (1.92%)	1 (1.92%)	0

*Plus sign indicates an increase in quantity and minus sign indicates a decrease in quantity.

The use of adalimumab increased because we recommended patients to replace inaccessible Remicade with other available biologics. We tried to develop personalized treatment plans for specific patients on time to minimize the impact of changes in medication regimens on the patient's disease condition during the epidemic. It was an important step to increase the self-management of IBD patients.

The result showed that 93.14% of patients had normal or even positive emotional states, which implied that the epidemic did not have a very negative impact on the mood of these IBD patients in such a relatively early month. However, since previous studies have shown that emotional stress is significantly associated with decreased quality of life [29, 30], it was very important to intervene in the emotional state in the process of IBD patient self-management guidance.

Limitations

The major limitation was that the sample size was small and our participants were not obtained by randomized sampling. In addition, there was no IBD patient diagnosed with COVID-19 in our study. Further researches need to be done to get more evidence about the susceptibility to SARS-CoV-2 in IBD patients and the clinical manifestations of IBD patients complicated with COVID-19.

CONCLUSION

In the middle of February, although more than half of IBD patients we studied in Hubei Province were in remission and possessed a good HRQoL, the outbreak of COVID-19 still had a certain impact on IBD patients in Hubei Province, such as worsening of their self-reported disease conditions and changes in their treatment options. These changes deserved attention to the impact of epidemics on IBD patients. In our survey, doctors used the questionnaire to assess patients' disease conditions, give timely feedback and suggestions to IBD patients. This method facilitated the effective self-management of patients under the circumstance of the COVID-19 outbreak.

MATERIALS AND METHODS

Study design and participants

This study was an online questionnaire survey among IBD patients from the region of Hubei Province. From February 18, 2020 to February 20, 2020, the questionnaire was administered to a sample of IBD patients with regular

follow-up in our IBD center. There were two items of the inclusion criteria for this study. One was that the patient (aging from 14 to 80) was established diagnosis of IBD for at least three months, another was that the patient is available to finish the online questionnaire (by Wechat, QQ, website, email) by himself or with the help of others. The exclusion criterion was that the patient is not able to finish the questionnaire. During this study, patients with IBD had been informed of the study's aim. An IBD specialist nurse is explicitly trained in this questionnaire contacted them to explain the study objectives. Participants completed the questionnaire with an online survey portal. They completed questionnaires voluntarily and independently, under uncompensated conditions. This study was approved by the National Health Commission of China and Ethics Commission of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Questionnaire design

The questionnaire was a validated 60-item questionnaire that assesses IBD disease activity across multiple domains, including the 6-point Mayo, HBI and SIBDQ (Supplementary File 1). HBI score ranges from 0 to 16 or more, and the highest score depends on the number of liquid stools per day. HBI scores of 0 and 4 are assigned to the remission phase; 5 to 7 are assigned to mildly active disease phase; 8 to 16 are assigned to moderately active disease phase; ≥17 are assigned to severely active disease phase [31, 32]. We used 6-point Mayo, which composed of the stool frequency, bleeding components. 6-point Mayo score of 0 and 1.5 are assigned to the remission phase; 1.5 to 2.5 are assigned to mildly active disease phase; 2.5 to 4.5 are assigned to moderately active disease phase; 8 to 4.5 are assigned to severely active disease phase [33, 34]. Total SIBDQ score ranges from 10 to 70, to find out how the patients have been feeling during the last two weeks. They will be asked about symptoms related to IBD diagnosis, as well as the general emotional status during the period and their attitude to the plague. Patients with a SIBDQ score of more than 50 are considered to have a good HRQoL [35–37].

In addition to the question about HBI, 6-point Mayo score and SIBDQ, the questionnaire included questions regarding the subjective feeling about their change in disease conditions and epidemiological history questions about COVID-19.

Statistical analysis

Data collection and its statistical analysis were carried out using the SPSS software system (SPSS for Windows, Version 23.0, SPSS Inc., Chicago). Data analysis excluded incomplete items. Categorical data obtained were presented as frequency counts and percentages. Median, range and frequency were used to describe the demographic, SIBDQ scores and clinical characteristics. Chi-square tests were used to test the significant difference of categorical variables between two groups, such as the participants' distribution in different disease active phases and HRQoL between UC and CD patients, with fisher exact test as appropriate. The logistic regression analysis was used to identify variables significantly associated with disease activity, and a multivariate model was built to assess the effect of each potential confounding factor and determined independent and significant factors associated with the disease index. The odds ratio (OR) was calculated to quantify the corresponding risk. For all analyses, $p<0.05$ was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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REFERENCES

1. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. Lancet. 2020; 395:470–73. https://doi.org/10.1016/S0140-6736(20)30185-9 PMID:31986257

2. The Lancet. Emerging understandings of 2019-nCoV. Lancet. 2020; 395:311. https://doi.org/10.1016/S0140-6736(20)30186-0 PMID:31986259

3. Chen S, Yang J, Yang W, Wang C, Bärnighausen T. COVID-19 control in China during mass population movements at New Year. Lancet. 2020; 395:764–66. https://doi.org/10.1016/S0140-6736(20)30421-9 PMID:32105609

4. Kennedy A, Gask L, Rogers A. Training professionals to engage with and promote self-management. Health Educ Res. 2005; 20:567–78. https://doi.org/10.1093/her/cyh018 PMID:15741189

5. Osman L. Guided self-management and patient education in asthma. Br J Nurs. 1996; 5:785–89. https://doi.org/10.12968/bjon.1996.5.13.785 PMID:8974523

6. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol. 2003; 3:521–33. https://doi.org/10.1038/nri1132 PMID:12876555

7. Robinson A, Thompson DG, Wilkin D, Roberts C, and Northwest Gastrointestinal Research Group. Guided self-management and patient-directed follow-up of ulcerative colitis: a randomised trial. Lancet. 2001; 358:976–81. https://doi.org/10.1016/S0140-6736(01)06105-0 PMID:11583752

8. Breitscheidel L, Stamenitis S. Using patient-reported outcome assessments in clinical practice and their importance in risk management. J Med Econ. 2009; 12:180–81. https://doi.org/10.3111/13696990903216278 PMID:19691445

9. Bradley C. Feedback on the FDA's February 2006 draft guidance on Patient Reported Outcome (PRO) measures from a developer of PRO measures. Health Qual Life Outcomes. 2006; 4:78. https://doi.org/10.1186/1477-7525-4-78 PMID:17029628

10. Black N, Jenkinson C. Measuring patients' experiences and outcomes. BMJ. 2009; 339:b2495. https://doi.org/10.1136/bmj.b2495 PMID:19574317

11. Elkjaer M, Shuhaibar M, Burisch J, Bailey Y, Scherfig H, Laugesen B, Avnstrøm S, Langholz E, O'Morain C, Lynge E, Munkholm P. E-health empowers patients with ulcerative colitis: a randomised controlled trial of the web-guided 'Constant-care' approach. Gut. 2010; 59:1652–61. https://doi.org/10.1136/gut.2010.220160 PMID:21071584

12. Acquadro C, Berzon R, Dubois D, Leidy NK, Marquis P, Revicki D, Rothman M, and PRO Harmonization Group. Incorporating the patient's perspective into drug development and communication: an ad hoc task force report of the Patient-Reported Outcomes (PRO) Harmonization Group meeting at the Food and Drug Administration, February 16, 2001. Value Health. 2003; 6:522–31. https://doi.org/10.1046/j.1524-4733.2003.65309.x PMID:14627058

13. Keefer L, Kiebles JL, Taft TH. The role of self-efficacy in inflammatory bowel disease management: preliminary validation of a disease-specific measure. Inflamm Bowel Dis. 2011; 17:614–20. https://doi.org/10.1002/ibd.21314 PMID:20848516

14. Mikocka-Walus A, Knowles SR, Keefer L, Graff L. Controversies Revisited: A Systematic Review of the Comorbidity of Depression and Anxiety with Inflammatory Bowel Diseases. Inflamm Bowel Dis. 2016; 22:752–62. https://doi.org/10.1097/MIB.0000000000000620 PMID:26841224

15. Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A, Al-Jabir A, Iosifidis C, Agha R. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). Int J Surg. 2020; 76:71–76. https://doi.org/10.1016/j.ijsu.2020.02.034 PMID:32112977

16. Guo YR, Cao QD, Hong ZS, Tan YY, Chen SD, Jin HJ, Tan KS, Wang DY, Yan Y. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. Mil Med Res. 2020; 7:11. https://doi.org/10.1186/s40779-020-00240-0 PMID:32169119

17. Tian S, Hu N, Lou J, Chen K, Kang X, Xiang Z, Chen H, Wang D, Liu N, Liu D, Chen G, Zhang Y, Li D, et al. Characteristics of COVID-19 infection in Beijing. J Infect. 2020; 80:401–06. https://doi.org/10.1016/j.jinf.2020.02.018 PMID:32112886

18. Mao R, Liang J, Shen J, Ghosh S, Zhu LR, Yang H, Wu KC, Chen MH, and Chinese Society of IBD, Chinese Elite IBD Union, Chinese IBD Quality Care Evaluation Center Committee. Implications of COVID-19 for patients with pre-existing digestive diseases. Lancet Gastroenterol Hepatol. 2020; 5:425–27. https://doi.org/10.1016/S2468-1253(20)30076-5 PMID:32171057

19. Shigemura J, Ursano RJ, Morganstein JC, Kurosawa M, Benedek DM. Public responses to the novel 2019 coronavirus (2019-nCoV) in Japan: mental health consequences and target populations. Psychiatry Clin Neurosci. 2020; 74:281–82. https://doi.org/10.1111/pcn.12988 PMID:32034840

20. Wang C, Pan R, Wan X, Tan Y, Xu L, Ho CS, Ho RC. Immediate Psychological Responses and Associated Factors during the Initial Stage of the 2019 Coronavirus Disease (COVID-19) Epidemic among the General Population in China. Int J Environ Res Public Health. 2020; 17:E1729. https://doi.org/10.3390/ijerph17051729 PMID:32155789

21. Allam Z, Jones DS. On the Coronavirus (COVID-19) Outbreak and the Smart City Network: Universal Data Sharing Standards Coupled with Artificial Intelligence (AI) to Benefit Urban Health Monitoring and Management. Healthcare (Basel). 2020; 8:E46. https://doi.org/10.3390/healthcare8010046 PMID:32120822

22. Barlow C, Cooke D, Mulligan K, Beck E, Newman S. A critical review of self-management and educational interventions in inflammatory bowel disease. Gastroenterol Nurs. 2010; 33:11–8. https://doi.org/10.1097/SGA.0b013e3181ca03cc PMID:20145446

23. Boye B, Lundin KE, Jantschek G, Leganger S, Mokleby K, Tangen T, Jantschek I, Pripp AH, Wojniusz S, Dahlstroem A, Rivenes AC, Benninghoven D, Hausken T, et al. INSPIRE study: does stress management improve the course of inflammatory bowel disease and disease-specific quality of life in distressed patients with ulcerative colitis or Crohn's disease? A randomized controlled trial. Inflamm Bowel Dis. 2011; 17:1863–73. https://doi.org/10.1002/ibd.21575 PMID:21287660

24. Ranjbaran Z, Keefer L, Stepanski E, Farhadi A, Keshavarzian A. The relevance of sleep abnormalities to chronic inflammatory conditions. Inflamm Res. 2007; 56:51–7. https://doi.org/10.1007/s00011-006-6067-1 PMID:17431741

25. Narula N, Fedorak RN. Exercise and inflammatory bowel disease. Can J Gastroenterol. 2008; 22:497–504. https://doi.org/10.1155/2008/785953 PMID:18478136

26. Ng V, Millard W, Lebrun C, Howard J. Low-intensity exercise improves quality of life in patients with Crohn's disease. Clin J Sport Med. 2007; 17:384–88. https://doi.org/10.1097/JSM.0b013e31802b4fda PMID:17873551

27. Lenti MV, Selinger CP. Medication non-adherence in adult patients affected by inflammatory bowel disease: a critical review and update of the determining factors, consequences and possible interventions. Expert Rev Gastroenterol Hepatol. 2017; 11:215–26. https://doi.org/10.1080/17474124.2017.1284587 PMID:28099821

28. Lamb CA, Kennedy NA, Raine T, Hendy PA, Smith PJ, Limdi JK, Hayee B, Lomer MC, Parkes GC, Selinger C, Barrett KJ, Davies RJ, Bennett C, et al, and IBD guidelines eDelphi consensus group. British Society of Gastroenterology consensus guidelines on the management of inflammatory bowel disease in adults. Gut. 2019 (Suppl 3); 68:s1–106. https://doi.org/10.1136/gutjnl-2019-318484 PMID:31562236

29. Slonim-Nevo V, Sarid O, Friger M, Schwartz D, Chernin E, Shahar I, Sergienko R, Vardi H, Rosenthal A, Mushkalo A, Dizengof V, Ben-Yakov G, Abu-Freha N, et al, and Israeli IBD Research Nucleus (IIRN). Effect of psychosocial stressors on patients with Crohn's disease: threatening life experiences and family relations. Eur J Gastroenterol Hepatol. 2016; 28:1073–81. https://doi.org/10.1097/MEG.0000000000000666 PMID:27203602

30. Slonim-Nevo V, Sarid O, Friger M, Schwartz D, Sergienko R, Pereg A, Vardi H, Singer T, Chernin E, Greenberg D, Odes S, Dotan I, Chowers Y, et al, and Israeli IBD Research Nucleus (IIRN). Effect of Social Support on Psychological Distress and Disease Activity in Inflammatory Bowel Disease Patients. Inflamm Bowel Dis. 2018; 24:1389–400. https://doi.org/10.1093/ibd/izy041 PMID:29893949

31. Greenberg D, Schwartz D, Vardi H, Friger M, Sarid O, Slonim-Nevo V, Odes S, and Israeli IBD Research Nucleus [IIRN]. Health-Related Utility Weights in a Cohort of Real-World Crohn's Disease Patients. J Crohn's Colitis. 2015; 9:1138–45. https://doi.org/10.1093/ecco-jcc/jjv167 PMID:26374662

32. Hüppe A, Langbrandtner J, Häuser W, Raspe H, Bokemeyer B. Validation of the "German Inflammatory Bowel Disease Activity Index (GIBDI)": An Instrument for Patient-Based Disease Activity Assessment in Crohn's Disease and Ulcerative Colitis. Z Gastroenterol. 2018; 56:1267–75. https://doi.org/10.1055/a-0605-4080 PMID:29742780

33. Bewtra M, Brensinger CM, Tomov VT, Hoang TB, Sokach CE, Siegel CA, Lewis JD. An optimized patient-reported ulcerative colitis disease activity measure derived from the Mayo score and the simple clinical colitis activity index. Inflamm Bowel Dis. 2014; 20:1070–78. https://doi.org/10.1097/MIB.0000000000000053 PMID:24810138

34. de Jong MJ, Huibregtse R, Masclee AAM, Jonkers DMAE, Pierik MJ. Patient-Reported Outcome Measures for Use in Clinical Trials and Clinical Practice in Inflammatory Bowel Diseases: A Systematic Review. Clin Gastroenterol Hepatol. 2018; 16:648–663.e3. https://doi.org/10.1016/j.cgh.2017.10.019 PMID:29074448

35. Gonczi L, Kurti Z, Verdon C, Reinglas J, Kohen R, Morin I, Chavez K, Bessissow T, Afif W, Wild G, Seidman E, Bitton A, Lakatos PL. Perceived Quality of Care is

Associated with Disease Activity, Quality of Life, Work Productivity, and Gender, but not Disease Phenotype: A Prospective Study in a High-volume IBD Centre. J Crohn's Colitis. 2019; 13:1138–47. https://doi.org/10.1093/ecco-jcc/jjz035 PMID:30793162

36. Hlavaty T, Persoons P, Vermeire S, Ferrante M, Pierik M, Van Assche G, Rutgeerts P. Evaluation of short-term responsiveness and cutoff values of inflammatory bowel disease questionnaire in Crohn's disease. Inflamm Bowel Dis. 2006; 12:199–204. https://doi.org/10.1097/01.MIB.0000217768.75519.32 PMID:16534421

37. Ananthakrishnan AN, Weber LR, Knox JF, Skaros S, Emmons J, Lundeen S, Issa M, Otterson MF, Binion DG. Permanent work disability in Crohn's disease. Am J Gastroenterol. 2008; 103:154–61. https://doi.org/10.1111/j.1572-0241.2007.01561.x PMID:18076736

SUPPLEMENTARY MATERIAL

Please browse Full Text version to see the data of Supplementary File 1.

Supplementary File 1. Questionnaire for follow-up of patients with inflammatory bowel disease during the outbreak of Corona Virus Disease 2019

Research Paper

A qualitative study of the vocational and psychological perceptions and issues of transdisciplinary nurses during the COVID-19 outbreak

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ABSTRACT

Background: Due to its high infectivity and concealment, the coronavirus disease 2019 (COVID-19) outbreak that occurred in Wuhan attracted global attention. A special nursing group of transdisciplinary nurses (TNs) who had not worked in respiratory medicine, infection departments, or emergency and intensive medicine but who accounted for a large proportion of all nurses also drew our attention. Few studies have examined this special group of TNs. Therefore, this study collected the experiences and views of TNs at the forefront of the COVID-19 outbreak to investigate their potential problems.

Results: Twenty-five TNs and 19 nurses with experience in infectious diseases (non-TNs) were enrolled in the study. Compared with non-TNs, TNs showed higher levels of perceived stress and relatively less perceived social support. For TNs, the ambiguous roles, transition of operating mode, unfamiliar work content, and reversal of their daily schedule were the most common vocational problems. Additionally, most TNs had psychological problems such as anxiety, pain and insomnia. The incomprehension of parents, concern for family members and long-term isolation were the most common causes of psychological stress.

Conclusion: This survey is the first to focus on the group of TNs at the forefront of the COVID-19 outbreak and to investigate their experiences, vocational issues and psychological stresses qualitatively and quantificationally. We found that TNs had more perceived stress and less perceived social support than non-TNs. The vocational and psychological issues of TNs should be highlighted. These findings identify important issues and offer insights into the underlying issues to help TNs ultimately win the battle against novel coronavirus epidemics.

Methods: Semi-structured and face-to-face individual interviews and quantitative assessments were conducted. The Braun Clarke Thematic Analysis method and the strategy outlined by Miles and Huberman were used in the data analysis process of the qualitative study. The perceived stress scale and perceived social support scale were utilized to quantificationally evaluate the perceived stress level and the amount of perceived social support. Both qualitative and quantitative methods were adopted to assess the vocational and psychological perceptions and issues.

INTRODUCTION

In recent decades, there have been a variety of global coronavirus outbreaks, including severe acute respiratory syndrome coronavirus (SARS-COV) and Middle East respiratory syndrome coronavirus (MERS-COV), which have brought serious losses to human society [1, 2]. The coronavirus disease 2019 (COVID-19) outbreak, which occurred in Wuhan, HuBei Province, spread rapidly throughout the country and quickly attracted global attention [3, 4]. Given the high infectivity and concealment surrounding this outbreak, the government of China quickly generated containment strategies and performed a series of measures in the

early stage of the outbreak. Throughout the defensive and therapeutic system, crucial roles were played by medical workers, a large proportion of whom were nurses. In this study, we employ semi-structured interviews in combination with a scale assessment to focus on a special group of nurses and investigate their experiences, issues and challenges during their frontline work against COVID-19.

As the "gatekeepers" of the health care system, nurses at the forefront of the COVID-19 outbreak played key roles in identifying suspected and confirmed COVID-19 patients by carefully evaluating disease manifestations and exposure history [5]. In addition, as "interrupters", they implemented and maintained high-quality infection control measures to control the spread of COVID-19 [3, 5]. Because of the large-scale outbreak, multi-disciplinary nurses from all over the country participated in epidemic prevention and control. A special nursing group of transdisciplinary nurses (TNs) who had not worked in respiratory medicine, infection departments, or emergency and intensive medicine but who accounted for a large proportion of all nurses attracted our attention [6].

A series of studies have highlighted the important roles of appropriate emotions and stress management, the satisfaction of basic needs, sufficient social support, clear task distribution and flexible working schedules on nurses' work and psychological stress [7–9]. High-frequency and high-intensity work, including close contact with patients, produces occupational hazards and psychological stress for nurses. However, most researchers have placed more emphasis on nurses with experience in infectious diseases (non-TNs), especially those working in emergency and intensive medicine [10–12]. As a result, existing studies on TNs' experiences during the COVID-19 pandemic are limited, and quantitative and qualitative studies are lacking. Given TNs' limited experience in nursing during infectious pandemics, we suspected that these nurses likely endured even greater vocational and psychological stress [13, 14]. Therefore, we designed the study to collect the experiences and views of TNs at the forefront of the COVID-19 outbreak and to evaluate their psychological stresses. The results will emphasize an important issue and offer insights into the underlying issues to help TNs ultimately win the battle against novel coronavirus epidemics. In the long run, these findings may help health care institutions prepare for future pandemics.

RESULTS

In the present research, we primarily focused on the group of TNs at the front line against the COVID-19 outbreak and investigated their experiences, vocational issues and psychological stresses. In this part, the interrater reliability showed at least substantial agreement for every theme (κ = 0.63-0.85).

Awareness of nurses' responsibilities and roles

When we asked the participants about the responsibilities and obligations of nurses in the face of sudden acute infectious diseases threatening public health, we heard many similar and unmistakable voices promoting "the Nightingale spirit". One of the participants said,

"From the first day I became a nurse, I was deeply conscious of my responsibility to heal the wounded and rescue the dying. In the face of sudden novel coronaviruses, the lives and health of the world's population are under serious threat. As an angel in white, I have to summon "the Nightingale spirit" and go to the front lines where I am most needed to treat patients using professional knowledge and skills."

In addition, all participating nurses described their roles in the COVID-19 outbreak. Most of them thought of themselves as nurses, friends or even family. They not only focused on physical fitness but also maintained the mental health of patients:

"First, I should assist doctors in treating patients and do my job well. Furthermore, most patients in isolation for a long time are bored, alone and scared. I should also be their friend and family to establish a harmonious and friendly relationship with them to help them maintain their mental health."

When the participants mentioned changes in perceptions of nurses' job responsibilities and obligations, some of them noted that they had obtained a more divine sense of purpose and would continue their future work with this sense of professional mission:

"In the past, I used to do my job well, stick to my post, or try to be a "five-star" nurse. But this outbreak has made me see that everyone has a responsibility in the face of the epidemic, and the numerous serious cases have made my sense of responsibility and mission stronger. Our essential work is to help patients alleviate pain. Facing great disaster, I should have great love!"

Other nurses thought that their increased experience and excellent professional skills; particularly techniques for dealing with acute respiratory infections, would be beneficial for further work:

"Although most nursing work in daily life is closely related to my primary major, mastering more comprehensive nursing skills is a better guarantee for

the safety of patients. I can more skillfully and unhurriedly face the outbreak of other acute diseases endangering public health."

Recognition of responsibilities of transdisciplinary nursing work

We asked the participants, "With regard to respiratory infections, as a transdisciplinary nurse, how do you think the responsibilities differ from your usual work?" Differences in working contents and working patterns were the most common answers. One of the nurses in the surgical system said,

"Surgical work is based on 'panic-mode', and the faster work pace and turnover of patients is also significant. In addition to the routine work, I have to leave time to deal with some emergencies. However, the work mode here is mainly 'process-based', and the patient's condition is more complex; the disease is relatively continuous, and the work pace is also slower."

Another nurse in another medical system said the work patterns were not exactly the same, with an obvious difference in the process of observing patients' condition:

"For patients infected with COVID-19, I prefer to monitor the basic vital signs, such as temperature, blood pressure, respiration and oxygen saturation. I need to be constantly vigilant and accurately judge the changes in patients' conditions, especially the transition from mild to severe."

We further investigated the challenges and problems produced by the transdisciplinary field in this epidemic prevention and control work, and we mainly heard three types of answers: acquiring new knowledge, enforcing new regulations and improving physical and psychological quality.

"Although I had learned nursing knowledge in different specialties before, with the update of knowledge and technology and in order to accurately treat patients, I need to relearn nursing knowledge about the COVID-19 outbreak. In addition, when facing large-scale respiratory infectious diseases, the work regulations are completely different from daily work, which also requires an adaptation process. Moreover, we went almost eight hours without eating and drinking in the isolation ward, so it is also a great test of physical quality and mental state. These [issues] were not encountered in my previous work."

There is no doubt that there are many risks in nursing work, and the risks for transdisciplinary nurses are even greater in the fight against the COVID-19 outbreak.

"What I shouldn't ignore is the risk of occupational exposure. Improper protection or careless operation will greatly increase the risk of infection. The unfamiliar operation of a protective suit can significantly increase the risk of infection. In addition, contradictions between patients and nurses remain. The increased workload and the adaptation to the new environment will lead to mental stress and physical fatigue; in this state, the quality of nursing work will also decline."

When we mentioned the new understanding of nursing risks and risk prevention during frontline work against the epidemic, one of the TNs highlighted the importance of protection awareness and standardized operations:

"Acute infectious diseases are highly contagious and carry a high risk of infection, especially for health care workers who are in direct contact with patients. We must achieve accurate and standard operations, such as environment disinfection, detail control and protective suit operation. In addition, we should have a scientific understanding of the disease, enhance the awareness of protection, and successfully popularize knowledge. Ideological vigilance, attention to work, ensure mental health and physical health."

Psychological problems caused by transdisciplinary work

TNs play key roles in fighting at the forefront against the new coronavirus. However, when faced with acute respiratory infections, they have a relative lack of experience, and their psychological burdens increase remarkably under intense working pressure. Close social attention to their mental health is needed. When we asked about psychological problems when they were confronted with tough issues, worked in a strange and specific environment, faced high morbidity and mortality and worked under enormous pressure, most TNs answered that they experienced anxiety, grief, pain and insomnia:

"The professional preparation of disinfection, the intervention in patients' psychological problems and the document records were almost daily tasks, but I was not familiar and hadn't been specifically trained. With the heavy workload every day, I sometimes felt anxious and had insomnia at night."

"The high work intensity in the isolation ward, the disordered internal clock, and the restrictions and challenges of protective clothing all led me to be distressed."

"Although facing life and death is common for nurses, I have never experienced so many deaths in the past.

There was a growing body of critical patients dying while other new patients were constantly transferred to the intensive care unit every day. I often felt exhausted or even powerless. Not only was I sad that I could not treat my patients, but I also was sorrowful that I was not clear how long this situation would last."

Given that the nursing areas are transdisciplinary, most of the participants' families like did not understand why they had to be sent to the front. Therefore, the concerns of their families increased their psychological stress to some extent. Some nurses said they mainly worried about their children and parents:

"When I told my parents that I was going to the front, they didn't object, but it was clear they were worried and kept asking me why the TNs have to charge up. Although I explained patiently, I still worried about them."

In some families, both members of a couple are medical workers and sign up to go to the front to treat patients. In addition to worrying about each other, they are concerned about their children's lives and safety:

"My husband is a doctor majoring in respiratory medicine, and facing the epidemic, he resolutely went to the front. Although I am a surgical nurse, I believe I can also make contributions to epidemic prevention and control. However, I still feel sorry and deeply miss my child."

The levels of perceived stress and perceived social supports

In addition, we conducted a quantitative comparison of the perceived stress levels and the amount of perceived social supports between TNs and non-TNs. The result of PSS showed TNs had the higher level of perceived stress, with the significantly higher perceived stress scores than non-TNs (9.88±2.12 vs. 2.58±3.65) (Supplementary Figure 2A and Table 1). Furthermore, in terms of the perceived social supports, TNs got the remarkably less scores of PSSS (71.72±3.29 vs. 78.68±2.45), which represented the lower level of the perceived social supports of TNs than non-TNs (Supplementary Figure 2B and Table 1).

DISCUSSION

This interview survey is the first to pay attention to TNs, who constitute a large proportion of all nurses at the front line against the COVID-19 outbreak, and to provide insight into their vocational and psychological issues caused by the transdisciplinary work. Based on a survey of 25 TNs and 19 non-TNs, higher perceived stress levels and less perceived social support were detected in the TN group. Following further interviews with TNs, we found that ambiguous roles, the transition of operating modes, unfamiliar work contents, the environment and intensity and the reversal of daily schedules were the most common vocational problems for TNs. Additionally, almost all of the TNs had psychological problems such as anxiety, grief, pain and insomnia. Unacceptable mortality and the resulting powerlessness, incomprehension of parents, concern for family members and long-term isolation were the most common causes of psychological stress. These findings are consistent with other studies investigating nurses in emergency departments [15]. However, TNs seem to suffer from more psychological stress.

From conventional nursing to risk-averse infection control, the transformation of responsibility is the first challenge for TNs [16]. Most TNs have never received training for acute respiratory infections, nor have they been exposed to similar tasks, such as environmental disinfection or special care paperwork. For example, the related high risk of infection among TNs is partly because they are trained to temporarily wear and remove protective equipment and are unfamiliar with their operation. Therefore, these nurses believe that improving the ability and experience of TNs and nursing students in epidemic prevention and control is necessary to face epidemic outbreaks. In addition, efficient and reasonable pre-job training is an effective way for TNs to more quickly adapt to epidemic prevention and control-related nursing work.

Many studies have shown that clear role recognition is an important prerequisite for better nursing work [17–19]. For example, Lam K. emphasized that detailed role classification was beneficial for improving work efficiency [20]. The present study showed that more than half of TNs play ambiguous roles, and most of them play the role of psychologists many times to assist patients with psychological disorders. To some extent, the ambiguous roles of TNs at the forefront of the epidemic resulted in vocational issues. The TNs in this study believed that although medical resources were scarce during the specific period of the outbreak of the new coronavirus, more detailed role classification, clearer role definitions and job descriptions, and appropriate suggestions for expanded responsibilities would be effective methods to alleviate role ambiguity and improve work efficiency.

An important but overlooked problem is the psychological issues of nurses on the front line of epidemics. Arnaud Duhoux [21] and Sarah K. Schäfer [22] summarized general mental health problems and posttraumatic stress symptoms as the two most common types of psychopathological issues among

Table 1. Results of PSS and PSSS.

Group	N	PSS		PSSS	
		Means±SD	95%CI	Means±SD	95%CI
Non-TNs	19	2.58±3.65	0.77-4.38	78.68±2.45	77.47-79.90
TNs	25	9.88±2.12	8.93-11.07	71.72±3.29	70.37-73.73
t	-	-7.585		7.556	
P	-	<0.001		<0.001	

All data were normally distributed.

CI: confidence interval, N: numbers, Non-TNs: nurses experienced in infectious diseases, PSS: perceived stress scale, PSSS: perceived social support scale, TNs: transdisciplinary nurses.

nurses. Unfavorable working hours, including long work weeks, night shifts, weekend work, and quick returns, severely affect biological rhythms and work-life balance [23]. Intensive job attributes, including long-term emergency situations and a fast working pace, lead to a constantly high-pressure state [15]. These job characteristics considerably increase the risk of general mental health problems, such as depression, anxiety, insomnia, pain, and grief [24]. Furthermore, the pandemic and the high number of sudden patient deaths can result in posttraumatic stress symptoms reflected in the four aspects of intrusion, avoidance, negative alterations in cognition and mood and alterations in arousal and reactivity [22]. Because of their long-term working experiences that involve intensive and specific work content on the front line of the epidemic, most non-TNs are more familiar with the situation and can readily accommodate it. In contrast, TNs who lack experience with this type of working schedule, environment and intensity on the front line have more difficulty adapting and consequently are more likely to develop psychological disorders.

Although the psychological stress of nurses has been demonstrated to be higher than that of other professions and although nursing is also a high-risk occupation for psychological disease, in the context of a large-scale epidemic of infectious diseases, more attention should be paid to the special group of TNs [25–27]. The present survey about the psychological stresses of TNs found that anxiety, pain, grief and insomnia were the most common psychological problems, which is similar to other nurse-related studies, but with different causes: (1) their colleagues may be infected, and the number of infected TNs is significantly higher than non-TNs, which leads to anxiety among the TNs; (2) TNs have not been in a closed working environment and worn protective suits for a long time, which greatly challenges their psychological and physical limits; (3) unfamiliar working modes and a lack of skill in the content of their work increase their psychological burden; (4) most TNs have difficulty accepting high mortality and helplessness in the face of the large number of severe patients; and (5) compared with non-TNs, TNs suffer from more pressure from their family, and combined with concerns about their family members, their psychological burdens are significantly increased.

The results of the study suggest that in addition to patients' mental disorders, more attention should be paid to the psychological health of nurses, especially TNs. We can establish a psychological consultation platform for medical workers and increase the rear security of front-line medical workers to reduce psychological pressure and maintain their mental health. Furthermore, entertainment and sports facilities, such as running and dancing, could be established, which would be helpful to adjust emotions and relieve pressure.

In the present study, we highlighted the existing issues of TNs at the front line of the COVID-19 outbreak and provided some insights to further address vocational problems and alleviate psychological stress. In subsequent work against pandemics, a more appropriate work schedule, effective pre-job training and more detailed role classification will ameliorate the related vocational issues. In addition, measurements such as psychological consultation platforms and entertainment and sports facilities should be provided to protect the psychological health of TNs.

The present results offer a new perspective on the group of TNs, evaluate the transdisciplinary deficiencies and address existing issues in the treatment of pandemic infectious diseases. However, some limitations remain to be further discussed. (1) Most enrolled TNs worked in the same hospital, which likely resulted in directivity caused by locality and reduced credibility and objectivity. (2) The sample size was relatively low, and a larger-scale survey might further enhance the practical value. In the future, more participants, including TNs and non-TNs from various hospitals, will be recruited to expand the study. (3) The quantifiable measurements are limited, and quantitative follow-up and assessments should be combined to more accurately identify the existing vocational and psychological issues caused by

the transdisciplinary work and further improve the validity and quality of the research.

CONCLUSION

This study aimed to investigate the existing vocational and psychological issues of TNs against the novel coronavirus and attempted to offer possibilities for this special nursing group. This is the first survey to focus on the group of TNs and to investigate their experiences and vocational and psychological problems during the COVID-19 outbreak. We found that TNs had higher perceived stress levels and less perceived social support. Ambiguous roles, unfamiliar work patterns, a lack of skill in the work content leading to higher infection rates among colleagues, and family factors are prominent problems. These findings provide important information and insights into the underlying issues to help TNs ultimately win the battle against novel coronavirus epidemics.

MATERIALS AND METHODS

Design

We conducted a qualitative study utilizing semi-structured and face-to-face interviews to investigate the experiences, vocational issues and psychological stresses of front-line nurses in the process of fighting against the COVID-19 outbreak. The qualitative descriptive method is usually employed to explore individual experiences, cognitions, and inclinations regarding a specific phenomenon [28]. The utilization of a qualitative descriptive method can promote understanding of the phenomenon by soliciting rich viewpoints and opinions from the perspective of participants [29]. Besides, the perceived stress scale (PSS) (Supplementary File 1) and perceived social support scale (PSSS) (Supplementary File 2) were employed to assess their perceived stress levels and the amount of perceived social support. PSS is a psychological instrument to measure nonspecific perceived stress, and PSSS contains seven-point Likert scale ranging from 'strongly disagree' to 'strongly agree'. The flowchart of the entire study, from the screening of eligible nurses to data collection and analysis, is illustrated in Figure 1.

Selection of participants

A purposeful sampling method was used in this study to recruit eligible participants. This sampling method is beneficial in helping researchers collect relevant and valuable information by identifying different participants [30]. In the selection of TNs, nurses were invited to participate in the study if they met the following criteria: (1) registered nurses who had not worked in respiratory medicine, infection departments, or emergency and intensive medicine; (2) nurses in the frontline hospital for COVID-19 in Hubei; (3) actively and directly provided care for patients; and (4) were willing to share their opinions and ideas. In contrast, eligible non-TNs were required to be registered nurses who had experience in respiratory medicine, infection departments, or emergency or intensive medicine. In addition, the non-TNs completed only some of the

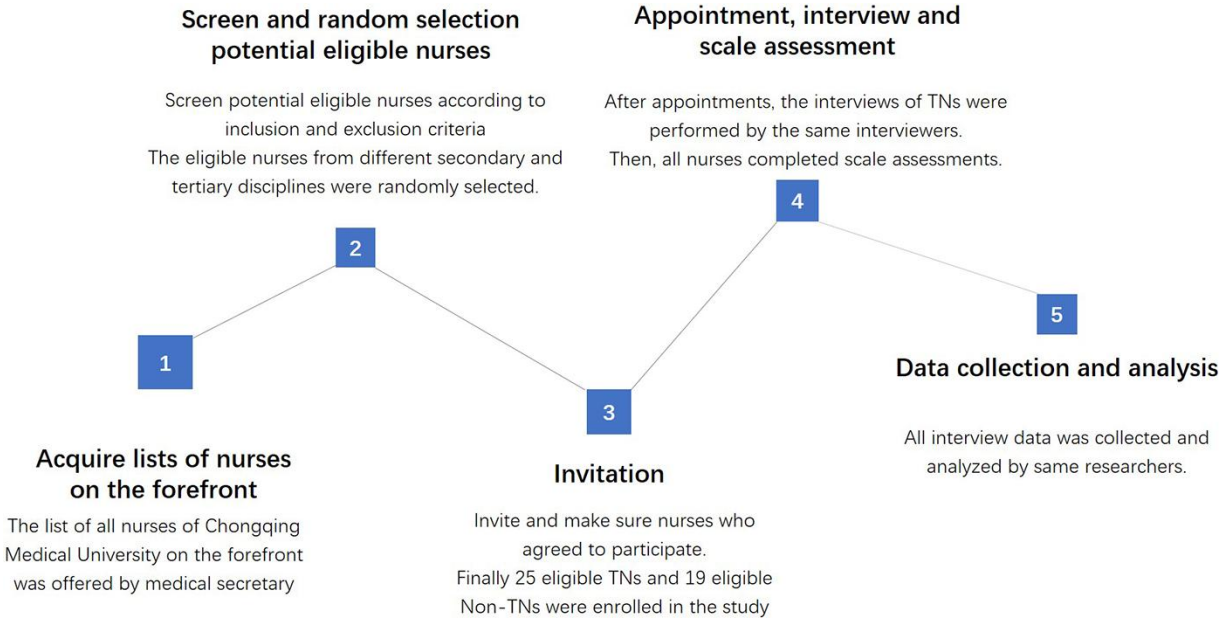


Figure 1. The flowchart of the interview study.

Table 2. Basic information.

Items	TNs [n(%), n=25]	Non-TNs [n(%), n=19]	P values
Gender			0.47
Female	21 (84)	17 (89.5)	
Male	4 (16)	2 (10.5)	
Age (years)			0.51
20-25	8 (32)	6 (31.6)	
26-30	9 (36)	8 (42.1)	
31-35	5 (20)	3 (15.8)	
36-40	2 (8)	2 (10.5)	
>40	1 (4)	0 (0)	
Job title			0.67
Nurse practitioner	20 (80)	15 (78.9)	
Supervisor nurse	5 (20)	4 (21.1)	
Work experience (years)			0.45
1-5	7 (28)	7 (36.8)	
6-10	11 (44)	8 (42.1)	
11-15	4 (16)	2 (10.5)	
>15	3 (12)	2 (10.5)	
Marital status			0.60
Single	6 (24)	4 (21.1)	
In love	2 (8)	2 (10.5)	
Ever-married	17 (68)	13 (68.4)	
Childbearing history			0.43
No children	9 (36)	8 (42.1)	
Be pregnant	0 (0)	0 (0)	
With children	16 (64)	11 (57.9)	

assessment scales. Because this study focused on the experience of front-line nurses in Hubei, nurses in management positions were excluded. Eligible individuals who were interested in participating in the study were contacted through email and were provided with detailed information on the research and the nature of their participation. Participants who were willing to participate in the study were asked to sign an informed consent form. Finally, 25 front-line TNs and 19 front-line non-TNs were enrolled in this study. Table 2 summarizes the demographic data of the participants. The demographic characteristics did not differ significantly between TNs and non-TNs. The various departments of the TNs and non-TNs are listed in Tables 3 and 4, respectively.

Ethical considerations

The research protocol was approved by the Ethics Committee of The First Affiliated Hospital of Chongqing Medical University. The study conformed to the ethical principles of medical research involving human subjects in the Helsinki Declaration [31]. The informed consent rights, privacy and anonymity of participants were protected.

Data collection

Semi-structured face-to-face interviews with the participants were conducted by the first author to solicit their experiences, vocational issues and psychological states in the forefront of the COVID-19 outbreak. The interviews were arranged in a convenient place for the participants, such as the lounge. To facilitate the follow-up data analysis, all interviews were recorded and backed up with the permission of the participants. The participants were encouraged to express their views and opinions freely. An interview guide comprising open-ended questions was utilized to lead the conversations to the study areas [32] (Figure 2 and Supplementary Figure 1). The average time for an interview was 45 minutes, ranging from 30 to 60 minutes.

In the scale assessments, all participants completed the PSS and the PSSS. The PSS was the version reorganized by Mota-Cardoso et al. [33], consisting of

Table 3. Different departments of TNs.

Departments	Results [n(%), n=25]
Surgical Department	
General Surgery	5 (20)
Urology	2 (8)
Orthopedics	2 (8)
Neurosurgery	1 (4)
Cardiothoracic Surgery	1 (4)
Internal Medicine	
Vasculocardiology	2 (8)
Gastroenterology	2 (8)
Endocrine Medicine	2 (8)
Nephrology	1 (4)
Hematopathology	1 (4)
Obstetrics and Gynaecology	1 (4)
Gerontology	1 (4)
Neurology	1 (4)
Dermatology and Venereology	**1 (4)**
Oncology	1 (4)
Otolaryngology	1 (4)

Table 4. Different departments of Non-TNs.

Departments	Results [n(%), n=19]
Respiratory medicine	5 (26.3)
Infections department	4 (21.1)
Emergency and intensive medicine	10 (52.6)

14 items with 5 alternatives per item, ranging from 0 to 4 points. The points indicated how often they felt or thought about certain events in the past month, from *never* (1 point) to *very often* (4 points). The internal consistency of PSS has been verified, with Cronbach's alpha = 0.90 in the study. The PSSS was composed of 12 items including the aspects of family, friend and others. Participants responded to the items on a 7-point scale representing the degree of agreement, form v*ery strongly disagree* (1 point) to *very strongly agree* (7 points). The internal consistency reliability coefficient of the PSSS was 0.91 in the present study. All

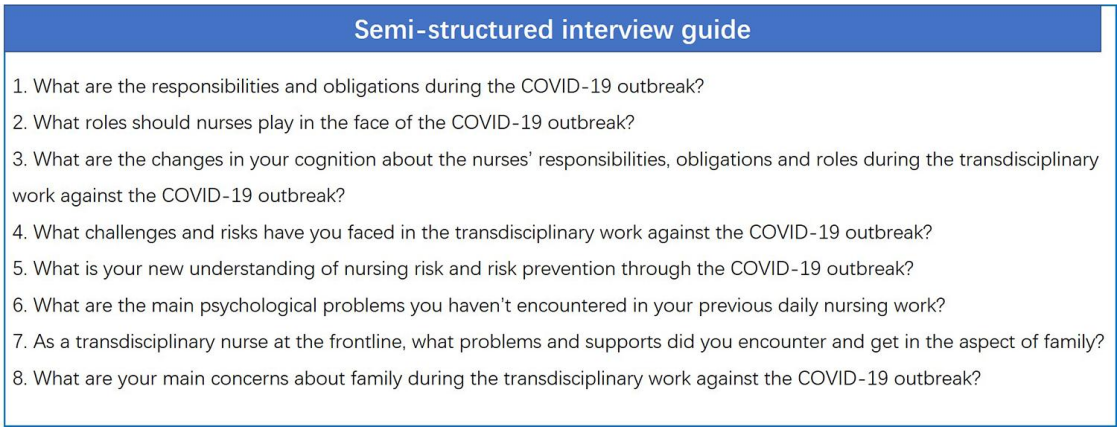


Figure 2. The semi-structured interview guide.

participants completed the scales in a lounge and the whole process of filling in the two scales took around 40 minutes. Subsequently, three researchers simultaneously converted the results of the paper scales to the online version of the scales.

Data analysis

Before the final data analysis of the interviews, the contents of each interview were recorded verbatim at the end of the day. All records were checked to ensure the accuracy of the transcription. Each record was analyzed within three days after the end of the corresponding interview. The Braun Clarke Thematic Analysis method and the strategy outlined by Miles and Huberman were used in the data analysis process [34, 35].

At the beginning of the data analysis process, all seven reliable researchers repeatedly read the interview records line by line and paragraph by paragraph to become familiar with the contents of the data. To develop preliminary codes, the narratives that were considered to be related to the phenomena in the study were emphasized. The records were scrutinized, and the relevant codes were further classified to form themes. Themes were also generated by codes that organized all of the data. We then reviewed these themes to refine the framework within and between themes to establish a network of themes and subthemes (Supplementary Table 1)**.**

The data dependability was established by checking codes among all researchers according to the strategy outlined by Miles and Huberman. All records were double coded by the research team. In all cases, we reached at least 80% agreement in assigning codes between two researchers. Disagreements were resolved by further discussion among the researchers. The investigator triangulation method enhanced the trustworthiness of the results.

Before analyzing the data of the PSS and PSSS, all paper scales were manually entered into the online scale version by three researchers simultaneously. When identical and credible results were obtained from the three researchers, the relevant data were further analyzed by SPSS (version 24). All quantitative data were normally distributed. MANOVAs, chi-square tests and t-tests for independent samples were employed to assess differences between non-TNs and TNs.

Trustworthiness

Trustworthiness is the standard that constitutes the rigor of qualitative research [36]. The trustworthiness of this study was maintained by establishing four main standards, including credibility, confirmability, transferability and dependability. In terms of credibility, the content of the study was discussed through continuous communications between the researchers and the supervisor. The supervisor conducted a critical assessment to identify defects in the investigation and corrected them with the researchers. In terms of confirmability, member-checking with all participants was completed to validate the interpreted findings [37]. Participants were asked to verify the survey results to ensure that their opinions were accurately reflected in the data and to check the consistency between the results of the researchers and the actual intentions of the participants. In terms of transferability, we used a vivid description method to ensure sufficient and accurate contextual information. The findings and conclusions can be transferred to other studies with similar situations [38].

Dependability is achieved through the accurate records and the in-depth description of the methods used in the research. Besides, Cohen's weighted kappa was employed to evaluate the interrater reliability. The poor agreement was considered if $\kappa < 0.00$, slight agreement if between 0.00 and 0.20, fair agreement if between 0.21 and 0.40, moderate agreement of between 0.41 and 0.60, substantial agreement if between 0.61 and 0.80, and almost perfect agreement of between 0.81 and 1.00 [39].

CONFLICTS OF INTEREST

These authors declare no conflicts of interest.

REFERENCES

1. Hsu CC, Chen T, Chang M, Chang YK, and CC. Confidence in controlling a SARS outbreak: experiences of public health nurses in managing home quarantine measures in Taiwan. Am J Infect Control. 2006; 34:176–81. https://doi.org/10.1016/j.ajic.2005.11.008 PMID:16679173

2. Holroyd E, McNaught C. The SARS crisis: reflections of Hong Kong nurses. Int Nurs Rev. 2008; 55:27–33. https://doi.org/10.1111/j.1466-7657.2007.00586.x PMID:18275532

3. Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A, Al-Jabir A, Iosifidis C, Agha R. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). Int J Surg. 2020; 76:71–76. https://doi.org/10.1016/j.ijsu.2020.02.034 PMID:32112977

4. Shi H, Han X, Jiang N, Cao Y, Alwalid O, Gu J, Fan Y, Zheng C. Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study. Lancet Infect Dis. 2020; 20:425–34.

https://doi.org/10.1016/S1473-3099(20)30086-4 PMID:32105637

5. Catton H. Global challenges in health and health care for nurses and midwives everywhere. Int Nurs Rev. 2020; 67:4–6. https://doi.org/10.1111/inr.12578 PMID:32083728

6. Hickey KT. Developing and Sustaining a Career as a Transdisciplinary Nurse Scientist. J Nurs Scholarsh. 2018; 50:20–27. https://doi.org/10.1111/jnu.12359 PMID:29135066

7. Petzold MB, Plag J, Ströhle A. Umgang mit psychischer Belastung bei Gesundheitsfachkräften im Rahmen der Covid-19-Pandemie [Dealing with psychological distress by healthcare professionals during the COVID-19 pandemia]. Nervenarzt. 2020; 91:417–21. https://doi.org/10.1007/s00115-020-00905-0 PMID:32221635

8. Mo Y, Deng L, Zhang L, Lang Q, Liao C, Wang N, Qin M, Huang H. Work stress among Chinese nurses to support Wuhan in fighting against COVID-19 epidemic. J Nurs Manag. 2020. [Epub ahead of print]. https://doi.org/10.1111/jonm.13014 PMID:32255222

9. Yifan T, Ying L, Chunhong G, Jing S, Rong W, Zhenyu L, Zejuan G, Peihung L. Symptom Cluster of ICU Nurses Treating COVID-19 Pneumonia Patients in Wuhan, China. J Pain Symptom Manage. 2020. [Epub ahead of print]. https://doi.org/10.1016/j.jpainsymman.2020.03.039 PMID:32276095

10. Kirtil I, Akyuz N. Precautions Taken by Nurses about the Prevention of Hospital-Acquired Infections in Intensive Care Units. Pak J Med Sci. 2018; 34:399–404. https://doi.org/10.12669/pjms.342.14610 PMID:29805416

11. Jónasdóttir RJ, Jónsdóttir H, Gudmundsdottir B, Sigurdsson GH. Psychological recovery after intensive care: outcomes of a long-term quasi-experimental study of structured nurse-led follow-up. Intensive Crit Care Nurs. 2018; 44:59–66. https://doi.org/10.1016/j.iccn.2017.06.001 PMID:28739293

12. Santana RDS, Fontes FLL, Morais MJA, Costa GDS, da Silva RK, de Araújo CS, da Silva ALB, Pereira RIDN. Occupational stress among emergency and urgent care nurses at a public hospital in Teresina, Piaui, Brazil. Rev Bras Med Trab. 2020; 17:76–82. https://doi.org/10.5327/Z1679443520190295 PMID:32270107

13. Fawcett J. Thoughts about multidisciplinary, interdisciplinary, and transdisciplinary research. Nurs Sci Q. 2013; 26:376–79. https://doi.org/10.1177/0894318413500408 PMID:24085679

14. Mueller SK. Transdisciplinary Coordination and Delivery of Care. Semin Oncol Nurs. 2016; 32:154–63. https://doi.org/10.1016/j.soncn.2016.02.009 PMID:27137472

15. Bardhan R, Heaton K, Davis M, Chen P, Dickinson DA, Lungu CT. A Cross Sectional Study Evaluating Psychosocial Job Stress and Health Risk in Emergency Department Nurses. Int J Environ Res Public Health. 2019; 16:E3243. https://doi.org/10.3390/ijerph16183243 PMID:31487874

16. Lam SK, Kwong EW, Hung MS, Pang SM, Chien WT, and SK. Emergency nurses' perceptions of their roles and practices during epidemics: a qualitative study. Br J Nurs. 2019; 28:523–27. https://doi.org/10.12968/bjon.2019.28.8.523 PMID:31002559

17. Liu H, Liehr P. Instructive messages from Chinese nurses' stories of caring for SARS patients. J Clin Nurs. 2009; 18:2880–87. https://doi.org/10.1111/j.1365-2702.2009.02857.x PMID:19747256

18. Shiao JS, Koh D, Lo LH, Lim MK, Guo YL, and JS. Factors predicting nurses' consideration of leaving their job during the SARS outbreak. Nurs Ethics. 2007; 14:5–17. https://doi.org/10.1177/0969733007071350 PMID:17334166

19. Phillips KP, O'Sullivan TL, Dow D, Amaratunga CA, and KP. Infectious respiratory disease outbreaks and pregnancy: occupational health and safety concerns of Canadian nurses. Prehosp Disaster Med. 2011; 26:114–21. https://doi.org/10.1017/S1049023X11000100 PMID:21888731

20. Lam SK, Kwong EW, Hung MS, Chien WT, and SKK. Emergency nurses' perceptions regarding the risks appraisal of the threat of the emerging infectious disease situation in emergency departments. Int J Qual Stud Health Well-being. 2020; 15:e1718468. https://doi.org/10.1080/17482631.2020.1718468 PMID:31975652

21. Duhoux A, Menear M, Charron M, Lavoie-Tremblay M, Alderson M. Interventions to promote or improve the mental health of primary care nurses: a systematic review. J Nurs Manag. 2017; 25:597–607. https://doi.org/10.1111/jonm.12511 PMID:28782168

22. Schäfer SK, Lass-Hennemann J, Groesdonk H, Volk T, Bomberg H, Staginnus M, Brückner AH, Holz E, Michael T. Mental Health in Anesthesiology and ICU Staff:

Sense of Coherence Matters. Front Psychiatry. 2018; 9:440.
https://doi.org/10.3389/fpsyt.2018.00440
PMID:30283365

23. Barleycorn D. Awareness of secondary traumatic stress in emergency nursing. Emerg Nurse. 2019; 27:19–22.
https://doi.org/10.7748/en.2019.e1957
PMID:31475501

24. Siegrist J, Dragano N. [Psychosocial stress and disease risks in occupational life. Results of international studies on the demand-control and the effort-reward imbalance models]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2008; 51:305–12.
https://doi.org/10.1007/s00103-008-0461-5
PMID:18369565

25. Chou TL, Ho LY, Wang KY, Kao CW, Yang MH, Fan PL. Uniformed service nurses' experiences with the severe acute respiratory syndrome outbreak and response in Taiwan. Nurs Clin North Am. 2010; 45:179–91.
https://doi.org/10.1016/j.cnur.2010.02.008
PMID:20510703

26. Su TP, Lien TC, Yang CY, Su YL, Wang JH, Tsai SL, Yin JC. Prevalence of psychiatric morbidity and psychological adaptation of the nurses in a structured SARS caring unit during outbreak: a prospective and periodic assessment study in Taiwan. J Psychiatr Res. 2007; 41:119–30.
https://doi.org/10.1016/j.jpsychires.2005.12.006
PMID:16460760

27. Marjanovic Z, Greenglass ER, Coffey S. The relevance of psychosocial variables and working conditions in predicting nurses' coping strategies during the SARS crisis: an online questionnaire survey. Int J Nurs Stud. 2007; 44:991–98.
https://doi.org/10.1016/j.ijnurstu.2006.02.012
PMID:16618485

28. Kim H, Sefcik JS, Bradway C. Characteristics of Qualitative Descriptive Studies: A Systematic Review. Res Nurs Health. 2017; 40:23–42.
https://doi.org/10.1002/nur.21768
PMID:27686751

29. Bradshaw C, Atkinson S, Doody O. Employing a Qualitative Description Approach in Health Care Research. Glob Qual Nurs Res. 2017; 4:2333393617742282.
https://doi.org/10.1177/2333393617742282
PMID:29204457

30. Suen LJ, Huang HM, Lee HH. [A comparison of convenience sampling and purposive sampling]. Hu Li Za Zhi. 2014; 61:105–11.
https://doi.org/10.6224/JN.61.3.105
PMID:24899564

31. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013; 310:2191–94.
https://doi.org/10.1001/jama.2013.281053
PMID:24141714

32. McIntosh MJ, Morse JM, and MJ. Situating and Constructing Diversity in Semi-Structured Interviews. Glob Qual Nurs Res. 2015; 2:2333393615597674.
https://doi.org/10.1177/2333393615597674
PMID:28462313

33. Mota-Cardoso R, Araújo A, Ramos RC, Gonçalves G, Ramos MO. Stress nos Professores Portugueses Estudo IPSSO 2000. Porto, Portugal: Porto Editora; 2002.

34. Braun V, Clarke V. Using thematic analysis in psychology. Qual Res Psychol. 2006; 3:77–101.
https://doi.org/10.1191/1478088706qp063oa

35. Linda S. Lotto. Qualitative Data Analysis: A Sourcebook of New Methods: Matthew B. Miles and A. Michael Huberman. SAGE journal. 1986.
https://doi.org/10.3102/01623737008003329

36. Woodcock S. Why do renal nurses focus on issues of compliance when working in the rehabilitative HD setting? EDTNA ERCA J. 1999; 25:28–30.
https://doi.org/10.1111/j.1755-6686.1999.tb00009.x
PMID:10418374

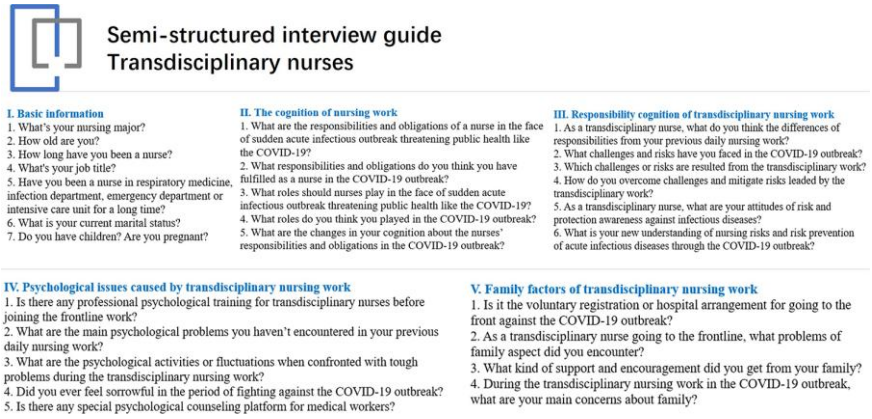
37. Lincoln Y, Guba E. Naturalistic Inquiry. Sage Publications; 1985.
https://doi.org/10.1016/0147-1767(85)90062-8

38. Pepler CJ, Edgar L, Frisch S, Rennick J, Swidzinski M, White C, Brown T, Gross J. Strategies to increase research-based practice: interplay with unit culture. Clin Nurse Spec. 2006; 20:23–31.
https://doi.org/10.1097/00002800-200601000-00008
PMID:16569946

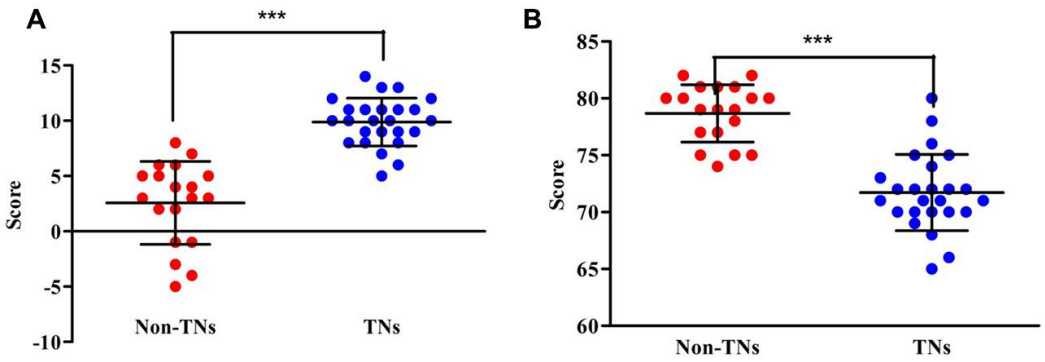
39. Landis JR, Koch GG, and JR. The measurement of observer agreement for categorical data. Biometrics. 1977; 33:159–74.
https://doi.org/10.2307/2529310
PMID:843571

SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. The semi-structural interview guide including five parts.



Supplementary Figure 2. The perceived stress levels and the amount of perceived social supports of TNs and Non-TNs. (**A**) the perceived stress scores, the higher score represents the higher level of perceived stress. (**B**) the perceived social support scores, the higher score represents the higher perceived social support level. The data are normally distributed, and are expressed as the means ± SD. ***P<0.001 TNs *vs.* Non-TNs.

Supplementary Table

Supplementary Table 1. The data record table.

Themes and Sub-themes	Results [n (%), n=25]
Responsibility cognition	
healing the wounded and rescuing the dying	25 (100)
the Nightingale spirit	19 (76)
win the battle against the COVID-19	18 (72)
relieve mental and psychological pressure of patients	15 (60)
take care of the daily life of patients	7 (28)
Role cognition	
nurse	25 (100)
friend	22 (88)
family	17 (68)
psychotherapist	13 (52)
patient care	5 (20)
New cognition of nursing work	
With new cognition	25 (100)
stronger sense of responsibility and mission	21 (84)
more comprehensive nursing skills	20 (80)
playing a variety of different roles	16 (64)
full of love to patients and the job	13 (52)
Without new cognition	0 (0)
Challenges of transdisciplinary nursing work	
unfamiliar working patterns	25 (100)
unfamiliar working contents	23 (92)
standardized professional operations	20 (80)
occupational exposure and self-protection	20 (80)
physical and psychological quality	19 (76)
Psychological issues	
grief	22 (88)
insomnia	18 (72)
anxiety	16 (64)
pain	13 (52)
depressed	7 (28)
dysphoria	4 (16)
Family factors of transdisciplinary nursing work	
miss and worry about families	25 (100)
parents don't understand	12 (48)
spouse doesn't understand	8 (32)
guilt towards families	5 (20)

Supplementary Files

Please browse Full Text version to see the data of Supplementary Files 1 and 2.

Supplementary File 1. Perceived Stress Scale.

Supplementary File 2. Multidimensional Scale of Perceived Social Support.

Research Paper

Epidemiological, comorbidity factors with severity and prognosis of COVID-19: a systematic review and meta-analysis

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ABSTRACT

A systematic review and meta-analysis was conducted in an attempt to systematically collect and evaluate the associations of epidemiological, comorbidity factors with the severity and prognosis of coronavirus disease 2019 (COVID-19). The systematic review and meta-analysis was conducted according to the guidelines proposed by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Sixty nine publications met our study criteria, and 61 studies with more than 10,000 COVID-19 cases were eligible for the quantitative synthesis. We found that the males had significantly higher disease severity (RR: 1.20, 95% CI: 1.13-1.27, P <0.001) and more prognostic endpoints. Older age was found to be significantly associated with the disease severity and six prognostic endpoints. Chronic kidney disease contributed mostly for death (RR: 7.10, 95% CI: 3.14-16.02), chronic obstructive pulmonary disease (COPD) for disease severity (RR: 4.20, 95% CI: 2.82-6.25), admission to intensive care unit (ICU) (RR: 5.61, 95% CI: 2.68-11.76), the composite endpoint (RR: 8.52, 95% CI: 4.36-16.65,), invasive ventilation (RR: 6.53, 95% CI: 2.70-15.84), and disease progression (RR: 7.48, 95% CI: 1.60-35.05), cerebrovascular disease for acute respiratory distress syndrome (ARDS) (RR: 3.15, 95% CI: 1.23-8.04), coronary heart disease for cardiac abnormality (RR: 5.37, 95% CI: 1.74-16.54). Our study highlighted that the male gender, older age and comorbidities owned strong epidemiological evidence of associations with the severity and prognosis of COVID-19.

INTRODUCTION

Since publicly characterized as a pandemic by the World Health Organization on March 11th, 2020, the coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2, has raised public concerns globally [1]. As of April 30th, 2020, it has caused 3,023,788 confirmed cases and 208,112 deaths [2]. Further, this number is expected to continue to grow rapidly for some time to come, and will threaten the lives, physical and mental health of more people worldwide [3]. To date, there are still no proven specific therapies

available for COVID-19, other than supportive cares [4, 5]. It's a matter of urgency that identifying potential factors affecting the severity and prognosis of COVID-19, and implementing individualized treatment, focused prevention and nursing.

Case-series or retrospective cohort studies have initially explored the associations of epidemiological, comorbidity factors with severity and prognosis of COVID-19 [the multi-stage endpoints including disease severity, acute respiratory distress syndrome (ARDS), an intensive care unit (ICU), the use of mechanical ventilation, or death, etc.] [6–13]. Huang et al. first explored the contribution of demographic and comorbidity factors for ICU admission in 41 COVID-19 cases, and got null results [6]. Further, they conducted a retrospective cohort study with 137 discharged and 54 patients who died, and concluded older age and comorbidities were associated with prognosis of COVID-19 [7]. Meanwhile, in another study with 201 patients, Wu et al. found that older age was associated with higher risk of ARDS and death [8]. A study with 1,590 patients revealed that comorbidities were associated with poorer clinical outcomes [9]. Of note, some studies reported inconsistent, even contradictory conclusions, which might be caused by limited sample size or low endpoint rate [10–13]. Besides, reporting of the same patients in different articles was another concern [14]. Against this context, a thorough understanding of the epidemiological and comorbidity factors upon COVID-19 is urgently warranted. Herein, a systematic review and meta-analysis was conducted to sought to collect and comprehensively evaluate the associations of epidemiological, comorbidity factors with the severity and prognosis of COVID-19.

RESULTS

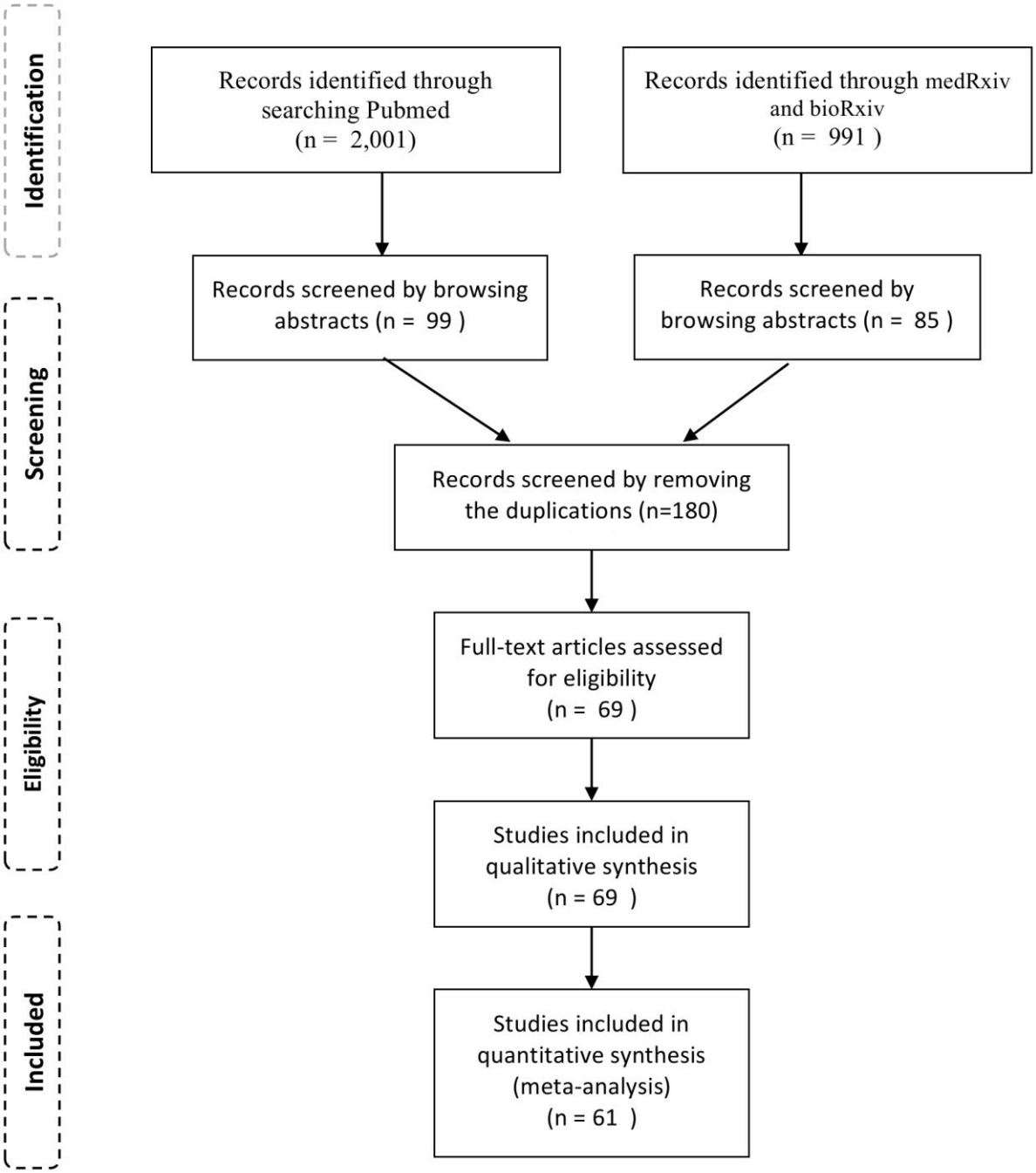
Study characteristics

Figure 1 presents the PRISMA flow diagram of this study. First, an initial search generated 2,992 potentially relevant papers, of which 2001 identified form Pubmed, and 991 from medRxiv or bioRxiv. After a number of screenings, 69 studies were identified (Supplementary Table 1). Of them, 67 (97.1%) reported Chinese COVID-19 patients, and 2 from either Japan or Singapore. The case number of each study ranged from 21 to 1780, with a mean of 218. The NOS score ranged from 5 to 7, which means a moderate methodological quality. Of the 69 publications, 2 duplicated studies (endpoints, exposure indicators and populations are completely covered by other studies), 4 studies with unique endpoints (survival ≤3d, refractory, liver injury, and time since symptom onset > 10 days), and 2 studies with different grouping methods for disease severity were excluded, which resulted that 61 studies were eventually eligible for the quantitative synthesis (Supplementary Table 1).

Quantitative data synthesis

The forest plots for all quantitative data synthesis of the epidemiological, comorbidity factors with severity and prognosis of COVID-19 were shown in supplementary materials (Supplementary Figures 1–120). Table 1 presents the quantitative results for the associations of the dichotomous epidemiological, comorbidity factors with severity of COVID-19. First, we found that the males had significant higher disease severity (RR: 1.20, 95% CI: 1.13-1.27, P <0.001, No. of cases: 8916). Besides, comorbidities, including any comorbidities, hypertension, diabetes, malignancy, cardiovascular disease, coronary heart disease, cerebrovascular disease, cardiovascular/cerebrovascular disease, chronic obstructive pulmonary disease (COPD), respiratory system disease, chronic kidney disease, hepatitis B infection, and digestive disease were significantly associated with the disease severity (all P <0.05). Of them, the top 3 effect sizes for the severity of COVID-19 were detected for COPD (RR: 4.20, 95% CI: 2.82-6.25, P<0.001), respiratory system disease (RR: 3.25, 95% CI: 2.48-4.27, P<0.001), and cerebrovascular disease (RR: 2.77, 95% CI: 1.70-4.52, P<0.001).

We also explored the associations of the dichotomous epidemiological, comorbidity factors with prognosis of COVID-19 (Supplementary Table 2, and Table 2). The males had higher risk of developing the endpoints including death, ARDS, admission to ICU, invasive ventilation, and cardiac abnormality. Hypertension was found to be associated with all seven endpoints, cardiovascular disease and cerebrovascular disease with 6 (except for disease progression), respiratory system disease with 6 (except for Cardiac abnormality), COPD with 5 (except for ARDS and cardiac abnormality), diabetes with 5 (except for the composite endpoint, cardiac abnormality), malignancy with 2 (death, and admission to ICU), etc. Among them, chronic kidney disease contributed mostly for death (RR: 7.10, 95% CI: 3.14-16.02, P<0.001), COPD for admission to ICU (RR: 5.61, 95% CI: 2.68-11.76, P<0.001), the composite endpoint (RR: 8.52, 95% CI: 4.36-16.65, P<0.001), invasive ventilation (RR: 6.53, 95% CI: 2.70-15.84, P<0.001), and disease progression (RR: 7.48, 95% CI: 1.60-35.05, P =0.011), cerebrovascular disease for ARDS (RR: 3.15, 95% CI: 1.23-8.04, P =0.016), coronary heart disease for cardiac abnormality (RR: 5.37, 95% CI: 1.74-16.54, P =0.003). Besides, the associations of continuous age with severity and prognosis of COVID-19 were presented in Table 3. Older age was found to be significantly associated with the disease severity and six endpoints (all P value <0.001, except a marginal



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For more information, visit www.prisma-statement.org.

Figure 1. PRISMA flow diagram.

Table 1. Quantitative data synthesis for the associations of the epidemiological, comorbidity factors with severity of COVID-19.

Variables	No of studies	Total cases	P heterogeneity	I^2 (%)	RR (95% CIs)	P value	P Egger
Sex, male	33	8916	0.078	27.2	1.20 (1.13-1.27)	<0.001	0.040
Smoking	11	5237	<0.001	80.8	1.56 (0.95-2.57)	0.082	0.956
Current smoking	2	2879	0.133	55.6	1.17 (0.92-1.50)	0.198	-
Ex-smoking	2	2879	0.019	81.7	2.17 (0.61-7.70)	0.232	-
Drinking	4	2274	0.067	58.0	0.83 (0.48-1.44)	0.516	0.722
Local residents of Wuhan	4	1931	<0.001	90.9	0.66 (0.32-1.36)	0.256	0.441
Exposure to Hubei Province	10	3127	<0.001	90.9	1.21 (0.88-1.65)	0.240	0.115
Contact with confirmed or suspect cases	13	5007	0.041	45.8	0.98 (0.86-1.13)	0.801	0.072
Family cluster	5	2578	0.857	0.0	0.94 (0.86-1.04)	0.224	0.856
Huanan seafood market exposure	5	2342	0.001	79.9	1.79 (0.38-8.35)	0.459	0.212
Comorbidities	16	6219	<0.001	83.4	1.72 (1.44-2.06)	<0.001	0.710
Hypertension	23	7739	<0.001	75.0	2.09 (1.74-2.52)	<0.001	0.154
Diabetes	23	7739	0.017	42.6	1.95 (1.60-2.36)	<0.001	0.272
Malignancy	14	5905	0.137	30.0	1.56 (1.11-2.21)	0.011	0.644
Cardiovascular disease	18	6841	0.019	45.5	2.74 (2.03-3.70)	<0.001	<0.001
Coronary heart disease	8	3899	0.087	43.7	2.03 (1.39-2.15)	<0.001	0.040
Cerebrovascular disease	12	5756	0.074	40.0	2.77 (1.70-4.52)	<0.001	0.595
Cardiovascular/cerebrovascular disease	6	3057	<0.001	84.0	2.31 (1.31-4.08)	0.004	0.502
COPD	14	6609	0.492	0.0	4.20 (2.82-6.25)	<0.001	0.580
Respiratory system disease	18	7522	0.661	0.0	3.25 (2.48-4.27)	<0.001	0.577
Chronic kidney disease	15	4861	0.173	25.5	2.27 (1.55-3.32)	<0.001	0.179
Chronic liver disease	11	3248	0.201	25.5	1.35 (0.89-2.05)	0.165	0.782
Hepatitis B infection	3	1710	0.448	0.0	2.69 (1.32-5.51)	0.007	0.735
Lithiasis	2	308	0.873	0.0	3.03 (0.73-12.58)	0.127	-
Autoimmune disease	5	2202	0.727	0.0	2.52 (0.80-7.90)	0.113	0.997
Abnormal lipid metabolism	4	2246	0.648	0.0	0.57 (0.26-1.25)	0.162	0.080
Digestive disease	5	1013	0.492	0.0	1.80 (1.13-2.87)	0.014	0.717
Thyroid disease	3	348	0.350	0.0	2.37 (0.66-8.50)	0.186	0.387
Tuberculosis	2	592	0.473	0.0	2.74 (0.72-10.4)	0.141	-
Nervous system disease	3	796	0.368	0.0%	1.64 (0.68-3.93)	0.270	0.160
Endocrine system disease	3	796	<0.001	89.6	3.09 (0.70-13.64)	0.136	0.622

Table 2. Quantitative data synthesis for the associations of the epidemiological, comorbidity factors with prognosis of COVID-19 (P value<0.05).

Variables	No of studies	Total cases	P heterogeneity	I^2 (%)	RR (95% CIs)	P value	P Egger
Death							
Sex, male	10	4214	0.443	0.0	1.23 (1.14-1.33)	<0.001	0.276
Comorbidities	8	4499	<0.001	88.7	1.68 (1.32-2.13)	<0.001	0.248
Hypertension	11	4860	<0.001	84.4	1.74 (1.31-2.30)	<0.001	0.418
Diabetes	10	4748	0.001	67.1	1.75 (1.27-2.41)	0.001	0.057
Malignancy	6	3978	0.262	22.8	3.09 (1.59-6.00)	0.001	0.006
Cardiovascular disease	11	4860	<0.001	75.9	2.67 (1.60-4.43)	<0.001	0.654
Coronary heart disease	5	2452	<0.001	87.7	3.16 (1.45-6.91)	0.004	0.435
Cerebrovascular disease	6	3771	0.457	0.0	4.61 (2.51-8.47)	<0.001	0.766
COPD	4	3677	0.279	22.0	5.31 (2.63-10.71)	<0.001	0.107
Respiratory system disease	7	4472	0.185	31.8	3.22 (2.12-4.90)	<0.001	0.761
Chronic kidney disease	5	2219	0.477	0.0	7.10 (3.14-16.02)	<0.001	0.772
Admission to ICU							
Sex, male	5	2224	0.011	69.6	1.29 (1.13-1.47)	<0.001	0.651

Comorbidities	5	3747	0.038	60.5	1.82 (1.45-2.29)	<0.001	0.646
Hypertension	5	3747	0.601	0.0	2.31 (1.97-2.70)	<0.001	0.312
Diabetes	5	3747	0.084	51.4	1.88 (1.10-3.23)	0.021	0.457
Malignancy	5	3747	0.427	0.0	2.52 (1.38-5.59)	0.003	0.158
Cardiovascular disease	5	3747	0.511	0.0	2.74 (1.92-3.92)	<0.001	0.692
Cerebrovascular disease	3	3508	0.349	4.9	5.12 (2.86-9.17)	<0.001	0.273
COPD	4	3549	0.800	0.0	5.61 (2.68-11.76)	<0.001	0.740
Respiratory system disease	4	3549	0.613	0.0	4.66 (2.59-8.40)	<0.001	0.637
Composite endpoint							
Smoking	2	2879	0.604	0.0	2.67 (1.91-3.73)	<0.001	-
Comorbidities	2	3370	<0.001	95.3	1.96 (1.06-3.60)	0.031	-
Hypertension	2	3370	0.011	84.5	2.20 (1.44-3.36)	<0.001	-
Cardiovascular disease	2	3370	0.927	0.0	3.09 (2.09-4.57)	<0.001	-
Coronary heart disease	2	3370	0.473	0.0	3.36 (2.15-5.25)	<0.001	-
Cerebrovascular disease	2	3370	0.225	32.0	4.10 (2.34-7.18)	<0.001	-
COPD	2	3370	0.185	43.0	8.52 (4.36-16.65)	<0.001	-
Respiratory system disease	2	3370	0.185	43.0	8.52 (4.36-16.65)	<0.001	-
ARDS							
Sex, male	3	2090	0.464	0.0	1.15 (1.01-1.30)	0.033	0.353
Hypertension	3	2090	0.377	0.0	1.90 (1.57-2.30)	<0.001	0.520
Diabetes	3	2090	0.068	62.9	3.07 (1.28-7.36)	0.012	0.066
Cardiovascular disease	3	2090	0.244	29.2	2.26 (1.43-3.58)	<0.001	0.422
Cerebrovascular disease	2	1889	0.152	51.2	3.15 (1.23-8.04)	0.016	-
Respiratory system disease	2	1889	0.303	5.6	2.44 (1.20-4.97)	0.014	-
Invasive ventilation							
Sex, male	2	1825	0.403	0.0	1.35 (1.11-1.64)	0.002	-
Family cluster	2	1825	0.646	0.0	1.58 (1.13-2.14)	0.006	-
Comorbidities	3	3415	0.005	81.2	1.83 (1.19-2.79)	0.006	0.569
Hypertension	3	3415	0.131	50.9	2.35 (1.92-2.89)	<0.001	0.366
Diabetes	3	3415	0.131	50.8	1.85 (1.24-2.76)	0.003	0.021
Cardiovascular disease	3	3415	0.844	0.0	2.90 (1.63-5.15)	<0.001	0.618
Cerebrovascular disease	2	3370	0.602	0.0	3.98 (1.77-8.93)	0.001	-
COPD	2	3370	0.383	0.0	6.53 (2.70-15.84)	<0.001	-
Respiratory system disease	3	3415	0.260	25.7	4.34 (2.04-9.26)	<0.001	0.567
Cardiac abnormality							
Sex, male	4	439	0.211	33.6	1.33 (1.02-1.72)	0.036	0.624
Hypertension	4	439	0.947	0.0	2.97 (1.65-5.34)	<0.001	0.610
Cardiovascular disease	4	439	0.915	0.0	4.90 (1.82-13.21)	0.002	0.177
Coronary heart disease	3	386	0.819	0.0	5.37 (1.74-16.54)	0.003	0.408
Disease progression							
Hypertension	2	219	0.547	0.0	2.90 (1.45-5.81)	0.003	-
Diabetes	2	219	0.746	0.0	3.30 (1.08-10.07)	0.036	-
COPD	2	219	0.848	0.0	7.48 (1.60-35.05)	0.011	-
Respiratory system disease	2	219	0.848	0.0	7.48 (1.60-35.05)	0.011	-

association for disease progression). The biggest standard mean difference (SMD) was detected for death (SMD: 1.06, 95% CI: 0.85-1.26, P<0.001). However, we didn't find any statistically significant associations for epidemiological factors, including drinking, local residents of Wuhan, exposure to Hubei Province, contact with confirmed or suspect cases, family cluster, and Huanan seafood market exposure. Sensitivity analyses by changing the pooling model and statistical variables, or using one-at-a-time method, were performed to assess the stability of the results. However, we found the results were not materially changed (data not shown). Further, we applied the Egger test to evaluated the potential publication bias, and very litter evidence (among all

Table 3. Quantitative data synthesis for the associations of age with severity and prognosis of COVID-19.

Variables	No of studies	Total cases	$P_{heterogeneity}$	I^2 (%)	SMD (95% CIs)	P value	P_{Egger}
Severity	32	8140	<0.001	92.4	0.73 (0.53-0.94)	<0.001	0.331
Death	9	3725	0.005	63.9	1.06 (0.85-1.26)	<0.001	0.610
Admission to ICU	5	2224	0.189	34.9	0.78 (0.60-0.96)	<0.001	0.538
Composite endpoint	2	2879	0.055	72.9	0.88 (0.56-1.21)	<0.001	-
ARDS	3	2090	0.939	0	0.83 (0.67-0.99)	<0.001	0.882
Invasive ventilation	2	1825	0.493	0.0	0.84 (0.54-1.14)	<0.001	-
Cardiac abnormality	4	439	0.041	63.6	0.92 (0.44-1.41)	<0.001	0.885
Disease progression	2	219	<0.001	95.4	2.37 (0.00-4.74)	0.050	-

120 associations, only 6 presented the existence of possible publication bias) was detected (Tables 1–3 and Supplementary Table 2).

DISCUSSION

To our knowledge, this should be the most comprehensive assessment of epidemiological, comorbidity factors with the severity and prognosis of COVID-19 conducted to date. We systematically evaluated data for more than ten thousand COVID-19 cases from 69 publications in the past several months, and identified that the males had higher risk of reaching severe disease and adverse prognostic endpoints. Older age was found to be significantly associated with the disease severity and six prognostic endpoints. Comorbidities, including hypertension, diabetes, cardiovascular disease, cerebrovascular disease, COPD, chronic kidney disease, and malignancy, contributed significantly to the disease severity and prognostic endpoints of COVID-19. Results from the current study would be helpful for implementing individualized treatment, focused prevention and nursing of COVID-19.

The "Gender and COVID-19 Working Group" first raised the concern of the gendered impacts of the COVID-19 outbreak [15], then echoed by another two publications [16, 17]. In a large epidemiological investigation in China with 72,314 cases, 51.0% of the patients were the males [18]. In a recent report of 1,590 hospitalized COVID-19 patients in China [9], the male rate was 57.3%, and it was 51.3% in Huoshenshan study with 1780 hospitalized cases. All these evidence indicated the almost equal sex distribution and disease susceptibility. Despite this, we identified that the males had higher rate of severity and prognostic endpoints in our meta-analysis. This finding was indirectly proved by a Italian study with 1591 ICU patients, the male rate of which was 82.0%, and higher than that previously reported [19]. However, the smoking status which has significant gender predisposition, showed no statistical associations with disease severity and prognosis of COVID-19, except for composite endpoint. The relationship between smoking and COVID-19 has become a very controversial topic, and should be interpreted with caution, as many factors could affect the results, such as the statistical power, definition of smoking status, the presence of confounding factors, and the potential role of angiotensin-converting enzyme-2 (ACE-2) [20–22]. Older age was another strong determinant of disease referral and outcomes in our results, which has been proved by a model-based analysis [23], and supported by studies of severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS) [24, 25].

In addition to epidemiological factors, comorbidities are also potentially important aspects which could affect the disease severity and prognosis of COVID-19. As a key regulator of blood pressure, angiotensin-converting enzyme (ACE) was also the binding site of SARS-CoV, making hypertension the most focused comorbidity [26, 27]. In our meta-analysis, we found hypertension was associated with higher rate of the disease severity and all prognostic endpoints. Of note, using of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II type 1 receptor blockers (ARBs) could contribute to the improvement of outcomes of COVID-19 patients with hypertension [28]. COPD was a major predominant indicator for the disease severity and prognosis of COVID-19. In our study, COPD contributed most to the admission to ICU, the composite endpoint, and invasive ventilation of COVID-19. Among the comorbidities, the contribution of malignancy to the prognosis of COVID-19 was a controversial topic. Liang et al. [29] first reported that patients with cancer had a higher risk of COVID-19 and with a poorer prognosis than those without cancer, then challenged by two other publications because of the sample size, and confounding factors [30, 31]. Our meta-analysis temporarily supported Liang's conclusion that malignancy contributed to death, and admission to ICU with a moderate sample size, although we can't adjusted for the potential confounding bias. An interesting finding

was that chronic kidney disease contributed mostly to the death. It is likely an immunologic explanation, given our current understanding of weakened immune system in patients with chronic kidney disease [32]. A more targeted and intensive health protection strategy for the patients with comorbidities above may be warranted.

The strengths of our study included an extensive systematic search strategy, a thorough examination of duplicate data, and a comprehensive quality assessment of the primary studies. The findings in the current study are also affected by several limitations. First, although we have systematically searched the literature to identify eligible studies, it is possible that some studies might have been missed. Despite the wide ranging search strategy, non-English language studies might not have been indexed in the databases we searched. Second, all studies except for two studies from Japan or Singapore, were from China in the early stage of the COVID-19 outbreak. This limited the findings' applicability across different populations and geographic regions during the COVID-19 pandemic. Third, although we have screened the hospital name, date of recruitment aiming to find duplicated usage of cases, it is inevitable as some studies used samples from multiple hospitals and have not reported detailed patient composition. This might affect the accurate estimates of disease prevalence or outcomes of COVID-19. Fourth, significant study heterogeneity or publication bias which may lead to questionable interpretation of result were detected for some associations (especially for comorbidities). Thus, these results should be interpreted with caution. Finally, moderate NOS score means the flawed methodological quality. However, as these studies were dealing with urgent public health concerns and carried out in a state of emergency, the quality was within acceptable limits. Taken together, despite some limitations, our study provides an important basis for a comprehensive understanding of disease severity and prognosis-related factors.

CONCLUSIONS

In our systematic review and meta-analysis, we highlighted that the male gender, older age and comorbidities showed strong epidemiological evidence of associations with the severity and prognosis COVID-19. Taken together, this large-scale meta-analysis not only summarizes the current literatures upon associations of epidemiological, comorbidity factors with the severity and prognosis of COVID-19, but also provides helpful clues for implementing individualized treatment, focused prevention and nursing of COVID-19. Further well-designed prospective cohort studies and randomized controlled trials are warranted to explore the severity and prognosis related factors of COVID-19.

MATERIALS AND METHODS

Search strategy and selection criteria

The systematic review and meta-analyses were conducted and reported according to the guidelines proposed by the PRISMA [33]. Studies were eligible for inclusion in this meta-analysis if they met the following criteria: (1) data published in a peer-reviewed journal in English or Chinese; (2) the study is a case-control, cohort, or a cross-sectional design in human beings; (3) the studies provide sufficient information for epidemiological, comorbidity factors with severity or prognosis of COVID-19; (4) When multiple publications reported on the same hospital, date of recruitment, exposures and endpoints, we defined it as duplicated studies. Some studies, although duplicate in terms of hospital and date of recruitment, were not judged to be duplicate because they evaluated different exposure indicators or endpoints.

Literature retrieval was conducted through a two-step strategy with a cut-off date of April 5th, 2020 (Figure 1). In step 1, we searched the PubMed database using the following key terms in combination: "2019-nCoV OR COVID-19 OR covid-2019 OR novel Coronavirus–Infected Pneumonia OR novel coronavirus OR SARS-CoV-2 OR Wuhan Coronavirus OR Wuhan pneumonia". In step 2, the COVID-19 or SARS-CoV-2 preprints were also retrieved from the medRxiv (https://www.medrxiv.org/) and bioRxiv (https://www.biorxiv.org/) databases using the above terms sequentially. We also searched the references and related articles of all gathered papers, and checked previously published meta-analyses and reviews. In the current study, we also incorporated the data of 1780 COVID-19 cases from Wuhan Huoshenshan hospital, the first and largest emergency specialty field hospital in epicenter Wuhan, China. Finally, 69 publications met our study criteria were included in the systematic review, and 61 studies were eligible for the quantitative synthesis.

Data extraction and quality control

All data were extracted by at least two authors (FX, LS, YH, and WP) according to the pre-specified selection criteria. Disagreement was resolved by discussion with a third party personnel. The details for each study including the first author, country, city or province, year of publication, source hospitals, date duration of the patient recruitment, PubMed identifier number (PMID) or the digital object identifier (DOI) number, total sample size, disease severity, clinical endpoints, the distribution of epidemiological and comorbidity factors, were extracted using a structured data sheet. Frequency numbers of dichotomous variables, and median (IQR,

interquartile range) or mean (SD, standard deviation) for continuous variables were recorded. Median (IQR) were transferred to the form of mean (SD) using the method recommended by the Cochrane handbook version 6, 2019. The endpoints consisted of disease severity, ARDS, admission to ICU, death, a composite endpoint, invasive ventilation, cardiac abnormality, and disease progression (the detailed definitions of the endpoints were presented in supplementary methods). The degree of severity of COVID-19 was determined using the American Thoracic Society guidelines for community-acquired pneumonia or the New Coronavirus Pneumonia Prevention and Control Guidelines of China [34, 35]. Two authors independently evaluated the methodological quality of included studies using the Newcastle-Ottawa Scale (NOS) for cohort study [36]. Any disagreement in the quality assessment was resolved by discussion with a third author. The NOS includes 8 items (up to 9 stars), each one of these items was scored from 0 to 1, except that a maximum of two stars can be given for comparability.

Data synthesis

All statistical analyses for this study were performed by STATA, version 12.0 (Stata Corporation, College Station, Texas). All tests were two-sided, and a *P*-value of less than 0.05 for any test or model was considered statistically significant unless otherwise stated. Meta-analysis was performed for all associations with data available from 2 or more independent samples. Summary relative ratios (RRs), or standard mean difference (SMD) with their 95% confidence intervals (CIs) were used to assess the strength of associations between epidemiological, comorbidity factors with the severity and prognosis of COVID-19 by either the fixed-effect model (Mantel–Haenszel method) or, in case of heterogeneity, the random-effect model (DerSimonian–Laird method). To assess inter-study heterogeneity, we calculated the chi-square-based Cochran's *Q* statistic test and I^2 statistic. Because of the low power of Cochran's *Q* statistic, heterogeneity was considered significant if $P < 0.10$. For I^2, values around 25% indicated low heterogeneity, around 50% moderate heterogeneity, and around 75% high heterogeneity. Publication bias was assessed visually by funnel plots and quantitatively with Egger' s regression test for asymmetry.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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REFERENCES

1. WHO. (2020). WHO Virtual press conference on COVID-19.

2. WHO. (2020). WHO COVID-19 Dashboard.

3. Bedford J, Enria D, Giesecke J, Heymann DL, Ihekweazu C, Kobinger G, Lane HC, Memish Z, Oh MD, Sall AA, Schuchat A, Ungchusak K, Wieler LH, and WHO Strategic and Technical Advisory Group for Infectious Hazards. COVID-19: towards controlling of a pandemic. Lancet. 2020; 395:1015–18. https://doi.org/10.1016/S0140-6736(20)30673-5 PMID:32197103

4. Kalil AC. Treating COVID-19-off-label drug use, compassionate use, and randomized clinical trials during pandemics. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.4742 PMID:32208486

5. FitzGerald GA. Misguided drug advice for COVID-19. Science. 2020; 367:1434. https://doi.org/10.1126/science.abb8034 PMID:32198292

6. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

7. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

8. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Zhou X, Du C, Zhang Y, Song J, Wang S, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. JAMA Intern Med. 2020. [Epub ahead of print]. https://doi.org/10.1001/jamainternmed.2020.0994 PMID:32167524

9. Guan WJ, Liang WH, Zhao Y, Liang HR, Chen ZS, Li YM, Liu XQ, Chen RC, Tang CL, Wang T, Ou CQ, Li L, Chen PY, et al. Comorbidity and its impact on 1590 patients

with COVID-19 in China: a nationwide analysis. Eur Respir J. 2020; 55:2000547. https://doi.org/10.1183/13993003.00547-2020 PMID:32217650

10. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

11. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, Li SB, Wang HY, Zhang S, Gao HN, Sheng JF, Cai HL, Qiu YQ, Li LJ. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-cov-2) outside of Wuhan, China: retrospective case series. BMJ. 2020; 368:m606. https://doi.org/10.1136/bmj.m606 PMID:32075786

12. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091. https://doi.org/10.1136/bmj.m1091 PMID:32217556

13. Qiu H, Wu J, Hong L, Luo Y, Song Q, Chen D. Clinical and epidemiological features of 36 children with coronavirus disease 2019 (COVID-19) in zhejiang, China: an observational cohort study. Lancet Infect Dis. 2020; 20:689–96. https://doi.org/10.1016/S1473-3099(20)30198-5 PMID:32220650

14. Bauchner H, Golub RM, Zylke J. Editorial concern-possible reporting of the same patients with COVID-19 in different reports. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.3980 PMID:32176775

15. Wenham C, Smith J, Morgan R, and Gender and COVID-19 Working Group. COVID-19: the gendered impacts of the outbreak. Lancet. 2020; 395:846–48. https://doi.org/10.1016/S0140-6736(20)30526-2 PMID:32151325

16. Bhopal R. Covid-19 worldwide: we need precise data by age group and sex urgently. BMJ. 2020; 369:m1366. https://doi.org/10.1136/bmj.m1366 PMID:32245830

17. Cai H. Sex difference and smoking predisposition in patients with COVID-19. Lancet Respir Med. 2020; 8:e20. https://doi.org/10.1016/S2213-2600(20)30117-X PMID:32171067

18. Epidemiology Working Group for NCIP Epidemic Response, Chinese Center for Disease Control and Prevention. [The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19) in China]. Zhonghua Liu Xing Bing Xue Za Zhi. 2020; 41:145–151. https://doi.org/10.3760/cma.j.issn.0254-6450.2020.02.003 PMID:32064853

19. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, Cereda D, Coluccello A, Foti G, Fumagalli R, Iotti G, Latronico N, Lorini L, et al, and COVID-19 Lombardy ICU Network. Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the lombardy region, Italy. JAMA. 2020; 323:1574–81. https://doi.org/10.1001/jama.2020.5394 PMID:32250385

20. Li Volti G, Caruso M, Polosa R. Smoking and SARS-CoV-2 disease (COVID-19): dangerous liaisons or confusing relationships? J Clin Med. 2020; 9:1321. https://doi.org/10.3390/jcm9051321 PMID:32370269

21. Lippi G, Sanchis-Gomar F, Henry BM. Active smoking and COVID-19: a double-edged sword. Eur J Intern Med. 2020; S0953-6205:30182–85. https://doi.org/10.1016/j.ejim.2020.04.060 PMID:32439289

22. Leung JM, Sin DD. Smoking, ACE-2, and COVID-19: ongoing controversies. Eur Respir J. 2020; 2001759. https://doi.org/10.1183/13993003.01759-2020 PMID:32430431

23. Verity R, Okell LC, Dorigatti I, Winskill P, Whittaker C, Imai N, Cuomo-Dannenburg G, Thompson H, Walker PG, Fu H, Dighe A, Griffin JT, Baguelin M, et al. Estimates of the severity of coronavirus disease 2019: a model-based analysis. Lancet Infect Dis. 2020; 20:669–77. https://doi.org/10.1016/S1473-3099(20)30243-7 PMID:32240634

24. Müller MA, Meyer B, Corman VM, Al-Masri M, Turkestani A, Ritz D, Sieberg A, Aldabbagh S, Bosch BJ, Lattwein E, Alhakeem RF, Assiri AM, Albarrak AM, et al. Presence of middle east respiratory syndrome coronavirus antibodies in Saudi Arabia: a nationwide, cross-sectional, serological study. Lancet Infect Dis. 2015; 15:559–64. https://doi.org/10.1016/S1473-3099(15)70090-3 PMID:25863564

25. Donnelly CA, Ghani AC, Leung GM, Hedley AJ, Fraser C, Riley S, Abu-Raddad LJ, Ho LM, Thach TQ, Chau P, Chan KP, Lam TH, Tse LY, et al. Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in hong kong. Lancet. 2003; 361:1761–66.

https://doi.org/10.1016/S0140-6736(03)13410-1
PMID:12781533

26. Turner AJ, Hiscox JA, Hooper NM. ACE2: from vasopeptidase to SARS virus receptor. Trends Pharmacol Sci. 2004; 25:291–94.
https://doi.org/10.1016/j.tips.2004.04.001
PMID:15165741

27. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. J Virol. 2020; 94:e00127–20.
https://doi.org/10.1128/JVI.00127-20
PMID:31996437

28. Meng J, Xiao G, Zhang J, He X, Ou M, Bi J, Yang R, Di W, Wang Z, Li Z, Gao H, Liu L, Zhang G. Renin-angiotensin system inhibitors improve the clinical outcomes of COVID-19 patients with hypertension. Emerg Microbes Infect. 2020; 9:757–60.
https://doi.org/10.1080/22221751.2020.1746200
PMID:32228222

29. Liang W, Guan W, Chen R, Wang W, Li J, Xu K, Li C, Ai Q, Lu W, Liang H, Li S, He J. Cancer patients in SARS-CoV-2 infection: a nationwide analysis in China. Lancet Oncol. 2020; 21:335–37.
https://doi.org/10.1016/S1470-2045(20)30096-6
PMID:32066541

30. Xia Y, Jin R, Zhao J, Li W, Shen H. Risk of COVID-19 for patients with cancer. Lancet Oncol. 2020; 21:e180.
https://doi.org/10.1016/S1470-2045(20)30150-9
PMID:32142622

31. Wang H, Zhang L. Risk of COVID-19 for patients with cancer. Lancet Oncol. 2020; 21:e181.
https://doi.org/10.1016/S1470-2045(20)30149-2
PMID:32142621

32. Sharma R, Kinsey GR. Regulatory T cells in acute and chronic kidney diseases. Am J Physiol Renal Physiol. 2018; 314:F679–98.
https://doi.org/10.1152/ajprenal.00236.2017
PMID:28877881

33. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. BMJ. 2009; 339:b2700.
https://doi.org/10.1136/bmj.b2700
PMID:19622552

34. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J, Crothers K, Cooley LA, Dean NC, Fine MJ, Flanders SA, Griffin MR, Metersky ML, Musher DM, et al. Diagnosis and Treatment of Adults with Community-acquired Pneumonia. An Official Clinical Practice Guideline of the American Thoracic Society and Infectious Diseases Society of America. Am J Respir Crit Care Med. 2019; 200:e45–e67.
https://doi.org/10.1164/rccm.201908-1581ST
PMID:31573350

35. Commission HC. (2020). The New Coronavirus Pneumonia Prevention and Control Guidelines of China.

36. Stang A. Critical evaluation of the newcastle-ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. Eur J Epidemiol. 2010; 25:603–05.
https://doi.org/10.1007/s10654-010-9491-z
PMID:20652370

SUPPLEMENTARY MATERIALS

Please browse Full Text version to see the data of Supplementary Methods, Figures and Tables related to this manuscript.

Research Paper

Risk factors influencing the prognosis of elderly patients infected with COVID-19: a clinical retrospective study in Wuhan, China

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ABSTRACT

The mortality rate of elderly patients with Coronavirus Disease 2019 (COVID-19) was significantly higher than the overall mortality rate. However, besides age, leading death risk factors for the high mortality in elderly patients remain unidentified. This retrospective study included 210 elderly COVID-19 patients (aged ≥ 65 years), of whom 175 patients were discharged and 35 died. All deceased patients had at least one comorbidity. A significantly higher proportion of patients in the deceased group had cardiovascular diseases (49% vs. 20%), respiratory diseases (51% vs. 11%), chronic kidney disease (29% vs. 5%) and cerebrovascular disease (20% vs. 3%) than that in the discharged group. The median levels of C-reactive protein (125.8mg/L vs. 9.3mg/L) and blood urea nitrogen (7.2mmol/L vs. 4.4mmol/L) were significantly higher and median lymphocyte counts (0.7×10^9/L vs. 1.1×10^9/L) significantly lower in the deceased group than those in the discharged group. The survival curve analysis showed that higher C-reactive protein (≥5mg/L) plus any other abnormalities of lymphocyte, blood urea nitrogen or lactate dehydrogenase significantly predicted poor prognosis of COVID-19 infected elderly patients. This study revealed that the risk factors for the death in these elderly patients included comorbidities, increased levels of C-reactive protein and blood urea nitrogen, and lymphopenia during hospitalization.

INTRODUCTION

Since the outbreak in December 2019, COVID-19 caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has rapidly grown into a pandemic worldwide [1–3]. While the overall mortality rate during the early phase of the pandemic in both China and Italy was around 2.3% [1, 4, 5], the mortality rate was significantly higher in the elderly especially in those aged 65 years or older. A report from the United States Centers for Disease Control and Prevention COVID-19 response team showed that 80% of deaths associated with COVID-19 were among adults aged ≥65 years [6], which is similar to that initially reported from China regarding the high mortality rate in elderly patients with COVID-19 [7, 8]. Available evidence

suggest that old age *per se*, especially aged ≥65 years [9], is an independent risk factor for COVID-19 related mortality irrespective of whether there may exist underlying comorbidities. In addition, the majority of the persons aged ≥65 years may have one or more pre-existing complications which would further increase the rate of mortality. At present, clinical research on COVID-19 has been mainly focused on the epidemiological characteristics, clinical manifestations, prognosis of the general population or comparisons between aged or young populations [9]. However, studies that specifically aimed to identify fatal risk factors for elderly COVID-19 patients (aged ≥65 years) are rare. Of note, a recent study showed that despite both age≥ 65 years and pre-existing comorbidities, including hypertension or diabetes, were independently associated with the development of acute respiratory distress syndrome (ARDS), age ≥ 65 years was a major risk factor associated with the progression from ARDS to death [9]. However, the main risk factors that are responsible for the death of elderly infected with SARS-CoV-2 have yet to be determined. Here, we report on the characteristics of the largest cohort of elderly COVID-19 patients in Wuhan city, China, the epicenter of the SARS-CoV-2 outbreak, and describe the fatal risk factors for the most fragile elderly patients with COVID-19 infection in the hope that this will help to guide the clinicians to identify the elderly people who are at higher danger to progress to severe illness from COVID-19 infection at an early stage and adjust treatment plans to reduce mortality.

RESULTS

Demographics and characteristics

A total of 302 patients aged ≥65 years old admitted between January 23, 2020 to February 29, 2020 were screened, and excluded 69 suspected cases admitted without laboratory confirmation tests for SARS-CoV-2, as were 9 cases transferred to Huoshenshan Hospital or Leishenshan Hospital, and 14 patients with only one laboratory test during hospitalization. Overall, 210 patients were finally included in this study (Table 1). The median age of the 210 patients was 71 (interquartile range [IQR] 67-77) years, and the ratio of male and female was approximate equal. 175 patients were in the discharged group, with a median age was 70 (IQR 67-74) years and 79 (45%) were male, while 35 patients were in the deceased group, with a median age was 74 (IQR 70-82) years, 22 (63%) were male. Patients in the deceased group were significantly older (74 years, IQR 70-82) than that in the discharged group (70 years, IQR 67-74). In the deceased group, the median time from onset of symptoms to admission and death were, respectively, 8 (IQR 6-14) days and 14 (IQR 12-24) days. In the discharged group, the median time from onset of symptoms to admission and discharge were 10 (IQR7-15) days and 26 (IQR21-29) days, respectively. A total of 18 cases required intensive care admission, with 16 cases in the deceased group.

Among the elderly patients, 159 (76%) had comorbidities, with hypertension (115 [55%]) and cardiovascular disease (52 [25%]) being the most common comorbidities, followed by diabetes (38 [18%]), respiratory disease (38 [18%]), and digestive disease (21 [10%]). There were 124 (71%) cases having comorbidities in the discharged group compared with 35 (100%) cases in the deceased group. Additionally, significantly higher percentage of patients in the deceased group had cardiovascular disease (17 [49%] vs. 35 [20%]), respiratory disease (18 [51%] vs. 20 [11%]), cerebrovascular disease (7 [20%] vs. 6 [3%]), chronic liver disease (6 [17%] vs. 12 [7%]), chronic kidney disease (10 [29%] vs. 8 [5%]) or malignancy (3 [9%] vs. 3 [2%]) than in the discharged group (Table 1).

Of the hospitalized elderly patients, the most common symptoms were fever (72%) and cough (71%), followed by chest stuffiness (36%), fatigue (35%), anorexia (11%), diarrhea (11%), pharyngalgia (10%), dyspnea (8%), headache (6%), myalgia (6%), and nausea or vomiting (5%). Among all the patients, half of them had fever (51%) as the first symptom, nearly one third had cough (31%), and a small proportion had pharyngalgia (8%), fatigue (4%), chest tightness (3%), diarrhea (2%), anorexia (0.5%), dyspnea (0.5%) as the first symptom.

Treatment and complications

Most of the elderly patients received antiviral therapy (90%), antibiotic therapy (82%), oxygen inhalation (67%), and one third of patients were treated with glucocorticoid (33%), part of the patients received gamma globulin therapy (19%), albumin therapy (11%), mechanical ventilation (11%) or continuous renal replacement therapy (CRRT) treatment (2%). Of all the patients, ARDS (13%, 27 of 210) was the most frequently complication, followed by acute renal failure (2%) and large cerebral infarction (1%) (Table 1). 71% (25/35) of patients in the deceased group developed ARDS as compared to 2 (1%) in the discharged group. And typical pulmonary Computed Tomographic (CT) changes from a deceased and a discharged patient were shown in Supplementary Figure 1A–1F.

Laboratory findings

Comparison of laboratory findings within 24 hours at admission were shown in Table 2. The median leucocyte counts (6.4×10^9/L) in patients in the deceased

Table 1. Baseline characteristics, treatments, complications of patients infected with COVID-1.

	Total (n=210)	Discharged group (n=175)	Deceased group (n=35)	P Value[a]
Age, median (IQR), y	71 (67-77)	70 (67-74)	74 (70-82)	<0.001
Sex, n (%)				
Male	101 (48)	79 (45)	22 (63)	0.056
Female	109 (52)	96 (55)	13 (37)	
Duration from onset of symptoms to admission, median (IQR), d	10 (7-15)	10 (7-15)	8 (6-14)	0.889
Duration from admission to outcome, median (IQR), d	14 (10-17)	14 (11-17)	9 (5-15)	<0.001
Duration from onset of symptoms to outcome, median (IQR), d	23 (17-28)	26 (21-29)	14 (12-24)	<0.001
ICU cases, n (%)	18/198 (9)	2/167 (1)	16/31 (52)	<0.001
Comorbidities, n (%)	159 (76)	124 (71)	35 (100)	<0.001
Hypertension	115 (55)	97 (55)	18 (51)	0.664
Diabetes	38 (18)	29 (17)	9 (26)	0.200
Cardiovascular disease	52 (25)	35 (20)	17 (49)	<0.001
COPD	3 (1)	2 (1)	1 (3)	0.435
Respiratory disease	38 (18)	20 (11)	18 (51)	<0.001
Cerebrovascular disease	13 (6)	6 (3)	7 (20)	<0.001
Chronic liver disease	18 (9)	12 (7)	6 (17)	0.047
Digestive diseases	21 (10)	15 (9)	6 (17)	0.123
Chronic kidney disease	18 (9)	8 (5)	10 (29)	<0.001
Malignancy	6 (3)	3 (2)	3 (9)	0.026
Number of Comorbidities, n (%)				
None	51 (24)	51 (29)	0 (0)	
One	56 (27)	48 (27)	8 (23)	
Two	60 (29)	54 (31)	6 (17)	
Three	22 (10)	16 (9)	6 (17)	<0.001
Four	13 (6)	4 (2)	9 (26)	
Five	4 (2)	1 (1)	3 (9)	
Six or more	4 (2)	1 (1)	3 (9)	
Signs and symptoms, n (%)				
Fever	151 (72)	122 (70)	29 (83)	0.115
Cough	148 (71)	118 (67)	30 (87)	0.031
Headache	13 (6)	10 (6)	3 (9)	0.523
Pharyngalgia	20 (10)	18 (10)	2 (6)	0.400
Fatigue	73 (35)	64 (37)	9 (26)	0.219
Anorexia	23 (11)	19 (11)	4 (11)	0.921
Nausea or vomiting	11 (5)	9 (5)	2 (6)	0.890
Myalgia	12 (6)	11 (6)	1 (3)	0.426
Chest stuffiness	76(36)	65(37)	11 (31)	0.522
Dyspnea	17 (8)	12 (7)	5 (14)	0.142
Diarrhea	24 (11)	21 (12)	3 (9)	0.562
First symptom, n (%)				
Fever	107 (51)	88 (50)	19 (54)	
Cough	65 (31)	55 (31)	10 (28)	
Pharyngalgia	16 (8)	14 (8)	2 (6)	
Fatigue	9 (4)	9 (5)	0 (0)	<0.001
Anorexia	1 (0.5)	1 (0.5)	0 (0)	
Chest tightness	6 (3)	4 (3)	2 (6)	
Dyspnea	1 (0.5)	1 (0.5)	0 (0)	
Diarrhea	5 (2)	3 (2)	2 (6)	
Temperature, °C	36.8 (36.5-37.0)	36.7 (36.5-36.9)	37.0 (36.5-37.8)	0.069
Heart rate, median (IQR), beat per minute	80 (78-88)	80 (78-86)	85 (80-104)	0.016

Respiratory rate, median (IQR), beat per minute	20 (20-22)	20 (20-22)	22 (20-26)	0.008
Mean arterial pressure, median (IQR), mm Hg	97 (92-105)	97 (93-104)	100 (92-109)	0.585
Treatment, n (%)				
Antiviral therapy	179/198 (90)	148/167 *(89)	31/31* (100)	0.048
Antibiotic therapy	163/198 (82)	133/167 (80)	30/31 (97)	0.022
Glucocorticoid therapy	65/198 (33)	42/167 (25)	23/31 (74)	<0.001
Gamma globulin therapy	37/198 (19)	23/167 (14)	14/31 (45)	<0.001
Albumin therapy	22/198 (11)	10/167 (6)	12/31 (39)	<0.001
Oxygen inhalation	133/198 (67)	103/167 (62)	30/31 (97)	<0.001
Mechanical ventilation	21/198 (11)	1/167 (1)	20/31 (66)	<0.001
CRRT	3/198 (2)	1/167 (1)	2/31 (7)	0.014
Complications, n (%)				
ARDS	27 (13)	2 (1)	25 (71)	<0.001
Acute renal failure	4 (2)	0 (0)	4 (11)	<0.001
Cerebral infarction	3 (1)	0 (0)	3 (9)	<0.001

Abbreviations: COVID-19, coronavirus disease 2019; IQR, interquartile range; ICU, intensive care unit; COPD, chronic obstructive pulmonary diseases; CRRT, continuous renal replacement therapy; ARDS, acute respiratory distress syndrome. [a] P values indicate differences between the discharged group and the deceased group. $P < 0.05$ was considered statistically significant. *Record/data were missing in 8 patients in the discharged group, and in 4 patients in the deceased group regarding whether or not the patients received a treatment.

group were higher than those in the discharged group (5.1×10^9/L). Among them, 9 (26%) patients had leucocytes counts above the normal range in the deceased group, compared with 3 (2%) in the discharged group. The concentrations of C-reactive protein in the deceased group were significantly higher than that in the discharged group, and the levels of C-reactive protein in deceased patients were all elevated beyond the normal range. The lymphocyte counts of the deceased group were progressively decreased compared with that of the discharged group, and 60% patients in the deceased group had lymphopenia while 18% patients in the discharged group had lymphopenia. The neutrophil counts in the deceased group were higher than that in the discharged group, and 43% cases in the deceased group had neutrophil counts above the normal range as compared to 6% in the discharged group. Compared with discharged group, the platelet counts were significantly lower in the deceased group.

Biochemical test results were shown in Table 2. The concentrations of aspartate aminotransferase (AST), serum creatinine (Cr), and blood urea nitrogen (BUN) of the deceased group were all significantly higher than that of the discharged group. And, nearly half (49%) of the deceased patients had BUN concentrations elevated beyond the normal range as compared to 11% of the patients in the discharged group. The levels of creatine kinase (CK) and creatine kinase isoenzyme (CK-MB) in the deceased group were significantly higher than those in the discharged group. Significantly more patients in the deceased group (86%) had procalcitonin concentrations above the normal range than in discharged group (21%, 35 of 170).

The concentrations of lactate dehydrogenase (LDH) were significantly higher in the deceased group than those in the discharged group, with 79% (27 of 34) deceased patients had LDH concentrations above the normal range as compared to 29% (50 of 171) in the discharged group. The median concentrations of fasting blood glucose did not differ significantly between the two groups, however relatively more patients in the deceased group (68%, 23 of 34) had acute hyperglycemia (glucose ≥6.1 mmol/L) as compared to 33% (57 of 171) in the discharged group. Albumin concentrations were significantly lower in the deceased group than in the discharged group, with 38% (13 of 34) deceased patients and 10% (17 of 172) discharged patients developed hypoalbuminemia. In addition, D-dimer level in the deceased patients was significantly higher than that in the discharged patients. The median activated partial thromboplastin time and prothrombin time as well as the total bilirubin concentrations in the deceased group were all significantly higher than those in the discharged group. Monocytes count, concentrations of alanine aminotransferase (ALT), triglyceride and thrombin time did not significantly differ between the two groups.

Receiver operating characteristic curve, survival curve and dynamic profile

The relationships between routine blood test results, including blood biochemistry, inflammatory markers

Table 2. Laboratory findings of patients infected with COVID-19.

Laboratory finding within 24 hours on admission	normal range	Median (IQR)			P value[a]
		Total (n=210)	Discharged group (n=175)	Deceased group (n=35)	
Leucocytes, $\times10^9$/L, n (%)	(3.5-9.5) $\times10^9$/L	5.2 (3.9-6.4)	5.1 (3.9-6.1)	6.4 (4.1-10.7)	0.037
<3.5 $\times10^9$/L		30 (14)	24 (14)	6 (17)	
3.5-9.5 $\times10^9$/L		168 (80)	148 (84)	20 (57)	<0.001
≥9.5		12 (6)	3 (2)	9 (26)	
C-reactive protein, mg/L, n (%)	(0-5) mg/L	15.5 (3.2-63.6)	9.3 (2.6-37.2)	125.8 (49.1-200.0)	<0.001
0-5 mg/L		68 (32)	68 (39)	0 (0)	<0.001
≥5 mg/L		142 (68)	107 (61)	35 (100)	
Lymphocyte, $\times10^9$/L, n (%)	(1.1-3.2) $\times10^9$/L	1.1 (0.8-1.5)	1.1 (0.9-1.6)	0.7 (0.4-0.9)	<0.001
<0.8 ×109/L		52 (25)	31 (18)	21 (60)	
0.8-1.1 ×109/L		42 (20)	35 (20)	7 (20)	<0.001
1.1-3.2×109/L		114 (54)	107 (61)	7 (20)	
≥3.2×109/L		2 (1)	2 (1)	0 (0)	
Neutrophils, $\times10^9$/L, n (%)	(1.8-6.3) $\times10^9$/L	3.3 (2.4-4.5)	3.0 (2.4-4.3)	5.2 (2.7-10.0)	0.003
<1.8 ×109/L		20 (10)	16 (9)	4 (11)	
1.8-6.3 ×109/L		165 (78)	149 (85)	16 (46)	<0.001
≥6.3 ×109/L		25 (12)	10 (6)	15 (43)	
NLR		2.9 (1.9-5.1)	2.8 (1.8-3.8)	8.4 (3.1-13.1)	<0.001
Monocytes, $\times10^9$/L	(0.1-0.6) $\times10^9$/L	0.4 (0.3-0.5)	0.4 (0.3-0.5)	0.4 (0.2-0.5)	0.225
Procalcitonin, ng/mL, n (%)	(0.02-0.05) ng/mL				
<0.02 ng/mL		0	0	0	
0.02-0.05 ng/mL		140/205 (68)	135/170 (79)	5 (14)	<0.001
≥0.05 ng/mL		65/205 (32)	35/170 (21)	30 (86)	
Platelet count, $\times10^9$/L, n (%)	(125-350) $\times10^9$/L	206.0 (159.8-267.0)	216.0 (169.0-285.0)	168.0 (124.0-216.0)	<0.001
<125 ×109/L		20/208 (10)	19/174 (11)	1/34 (3)	
125-350 ×109/L		164/208 (79)	139/174 (80)	25/34 (74)	0.010
≥350 ×109/L		24/208 (11)	16/174 (9)	8/34 (23)	
ALT, IU/L, n (%)	(7-40) IU/L	29.5 (17.0-45.5)	27.0 (17.0-43.0)	35.0 (18.0-77.0)	0.427
<7 IU/L		1/208 (0.5)	0/173 (0)	1 (3)	
7-40 IU/L		140/208 (67)	121/173 (70)	19 (54)	0.261
≥40 IU/L		67/208 (32.5)	52/173 (30)	15 (43)	
AST, IU/L, n (%)	(0-45) IU/L	26.0 (21.0-38.3)	25.0 (20.0-35.5)	45.0 (26.0-72.0)	0.008
<45 IU/L		165/208 (79)	149/173 (86)	16 (46)	<0.001
≥45 IU/L		43/208 (21)	24/173 (14)	19 (54)	
Cr, μmol/L, n (%)	(40-105) μmol/L	67.1 (56.5-84.1)	65.2 (55.2-80.2)	76.0 (64.0-107.4)	0.012
<40 μmol/L		2/208 (1)	1/173 (0.5)	1 (3)	
40-105μmol/L		197/208 (95)	167/173 (96.5)	30 (86)	0.139
≥105μmol/L		9/208 (4)	5/173 (3)	4 (11)	
BUN, mmol/L, n (%)	(3.1-7.2) mmol/L	4.7 (3.4-6.1)	4.4 (3.3-5.6)	7.2 (5.0-11.1)	<0.001
<3.1 mmol/L		28 (13)	26 (15)	2 (5)	
3.1-7.2 mmol/L		146 (70)	130 (74)	16 (46)	<0.001
≥7.2 mmol/L		36 (17)	19 (11)	17 (49)	
CK, IU/L, n (%)	(30-180) IU/L	65.0 (43.0-116.0)	60.0 (41.0-91.5)	137.0 (54.0-363.0)	0.008
<30 IU/L		19/208 (9)	16/173 (9)	3 (9)	
30-180 IU/L		162/208 (78)	144/173 (83)	18 (51)	<0.001
≥180 IU/L		27/208 (13)	13/173 (8)	14 (40)	
CK-MB, IU/L, n (%)	(0-25) IU/L	10.0 (7.0-13.0)	9.0 (7.0-12.0)	13.0 (8.0-19.0)	0.002
<25 IU/L		204/208 (98)	171/173 (99)	33 (94)	0.074
≥25 IU/L		4/208 (2)	2/173 (1)	2 (6)	
APTT, s	(21-35) s	27.6 (23.7-31.4)	27.3 (23.5-30.6)	31.3 (26.0-35.4)	0.012
Prothrombin time, s	(10-13) s	11.6 (11.0-12.4)	11.6 (10.9-12.3)	12.1 (11.5-12.8)	0.035
Thrombin time, s	(13-21) s	19.5 (16.4-22.2)	19.6 (14.9-22.3)	18.7 (17.4-21.4)	0.180
D-dimer, μg/mL, n (%)	(<0.5) μg/mL	0.6 (0.2-1.9)	0.6 (0.2-1.1)	3.6 (0.6-5.7)	<0.001

0-0.5 μg/mL		42/168 (25)	40/139 (29)	2/29 (7)	0.014
≥0.5 μg/mL		126/168 (75)	99/139 (71)	27/29 (93)	
Albumin, g/L, n (%)	(40-55) g/L	35.7 (31.9-39.3)	36.2 (32.8-39.6)	31.2 (26.7-34.6)	<0.001
<30 g/L		30/206 (15)	17/172 (10)	13/34 (38)	<0.001
Glucose, mmol/L, n (%)	(3.9-6.1) mmol/L	5.7 (4.7-7.4)	5.5 (4.7-6.9)	6.9 (5.0-7.9)	0.105
<3.9 mmol/L		2/205 (1)	2/171 (1)	0/34 (0)	<0.001
3.9-6.1 mmol/L		123/205 (60)	112/171 (66)	11/34 (32)	
≥6.1 mmol/L		80/205 (39)	57/171 (33)	23/34 (68)	
Total bilirubin, μmol/L	(2-21) μmol/L	8.9 (6.6-11.9)	8.4 (6.5-11.6)	9.6 (6.7-16.7)	0.043
Triglyceride, mmol/L	(0.5-1.72) mmol/L	1.2 (0.9-1.7)	1.2 (0.9-1.6)	1.3 (0.9-1.9)	0.635
Total cholesterol, mmol/L	(3.1-5.7) mmol/L	3.9 (3.3-4.6)	4.0 (3.5-4.6)	3.4 (2.8-4.2)	0.051
Lactate dehydrogenase, IU/L, n (%)	(114-240) IU/L	208.0 (165.8-270.8)	199.0 (164.0-244.0)	367.0 (251.0-547.0)	<0.001
<240 IU/L		128/205 (62)	121/171 (71)	7/34 (21)	<0.001
≥240 IU/L		77/205 (38)	50/171 (29)	27/34 (79)	

Abbreviations: COVID-19, coronavirus disease 2019; IQR, interquartile range; NLR, neutrophil-to-lymphocyte ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, serum creatinine; BUN, blood urea nitrogen; CK, creatine kinase; CK-MB, creatine kinase isoenzyme; APTT, activated partial thromboplastin time. [a] P values indicate differences between the discharged group and the deceased group. $P < 0.05$ was considered statistically significant.

and the prognosis were analyzed. As shown in Figure 1A, the values of area under curve (AUC) of C-reactive protein, lymphocytes, BUN, glucose, LDH, and neutrophil to lymphocyte ratio (NLR) were respectively 0.857, 0.214, 0.769, 0.660, 0.766, and 0.774. The optimal cut-off values of C-reactive protein, BUN, glucose, LDH and NLR were 63 mg/L, 6.1 mmol/L, 6.5 mmol/L, 265 IU/L, 6.48 respectively (Table 3). It showed that higher C-reactive protein, BUN, LDH and NLR on admission could significantly predict poor prognosis of COVID-19 infected elderly patients.

Survival curves derived from C-reactive protein, lymphocyte, BUN, glucose, LDH and NLR individually and from the frequency of abnormal findings in relation to C-reactive protein, lymphocyte, BUN, and LDH were shown in Figure 1B–1H. The survival rate was much higher in patients with normal values of C-reactive protein, LDH and NLR. Abnormally high levels of BUN and glucose were associated with lower survival rate. Patients suffered severe lymphopenia had decreased survival rate, and the lower the lymphocyte count the lower the survival rate. All the deceased patients had abnormally high C-reactive protein level plus at least one abnormal value of either lymphocyte, BUN or LDH at admission. And, all elderly patients that concomitantly had abnormally high C-reactive protein plus two abnormalities of lymphocyte, BUN or LDH were in the deceased group.

Dynamic profile of the three major findings/predictors (i.e. C-reactive protein, lymphopenia and BUN), were tracked from 24 hours at admission, during hospitalization and from the last laboratory findings before discharge or death, respectively. As shown in Figure 2, at admission, all deceased patients had markedly high level of C-reactive protein than that in the discharged patients. The level of C-reactive protein slightly reduced during the time impending death, however, it was still higher than that in the discharged patients. Most deceased patients had lymphopenia at admission, and lymphopenia became more serious when approaching death. By contrast, few discharged patients had lymphopenia, and it returned to normal during hospitalization. At admission, most deceased patients had BUN above normal range as compared to that in the discharged patients, and the BUN levels increased when impending death (Figure 2).

DISCUSSION

This study, to our knowledge, is the largest cohort study to date of elderly COVID-19 patients with definitive outcomes of the disease and describes the fatal risk factors for the most fragile elderly patients. In keeping with the findings that aging is a risk factor for patients with COVID-19 in the overall population, the median age of patients in the deceased group was significantly older than the discharged group, suggesting that the older the patients, the higher the mortality. Consistently, epidemiological studies conducted among 72,314 patients across the China showed that the mortality of patients aged 70-79 years was 8.0%, and the mortality of patients aged over 80 years was 14.8% [7]. The relatively high mortality of elderly patients in our study (16.7%, 35/210) was possibly related to the lack of medical resources caused by the outbreak of COVID-19 initially in Hubei Province, China, and similarly in Europe and north America later on. But, most likely, the high mortality in the elderly may be attributable to the

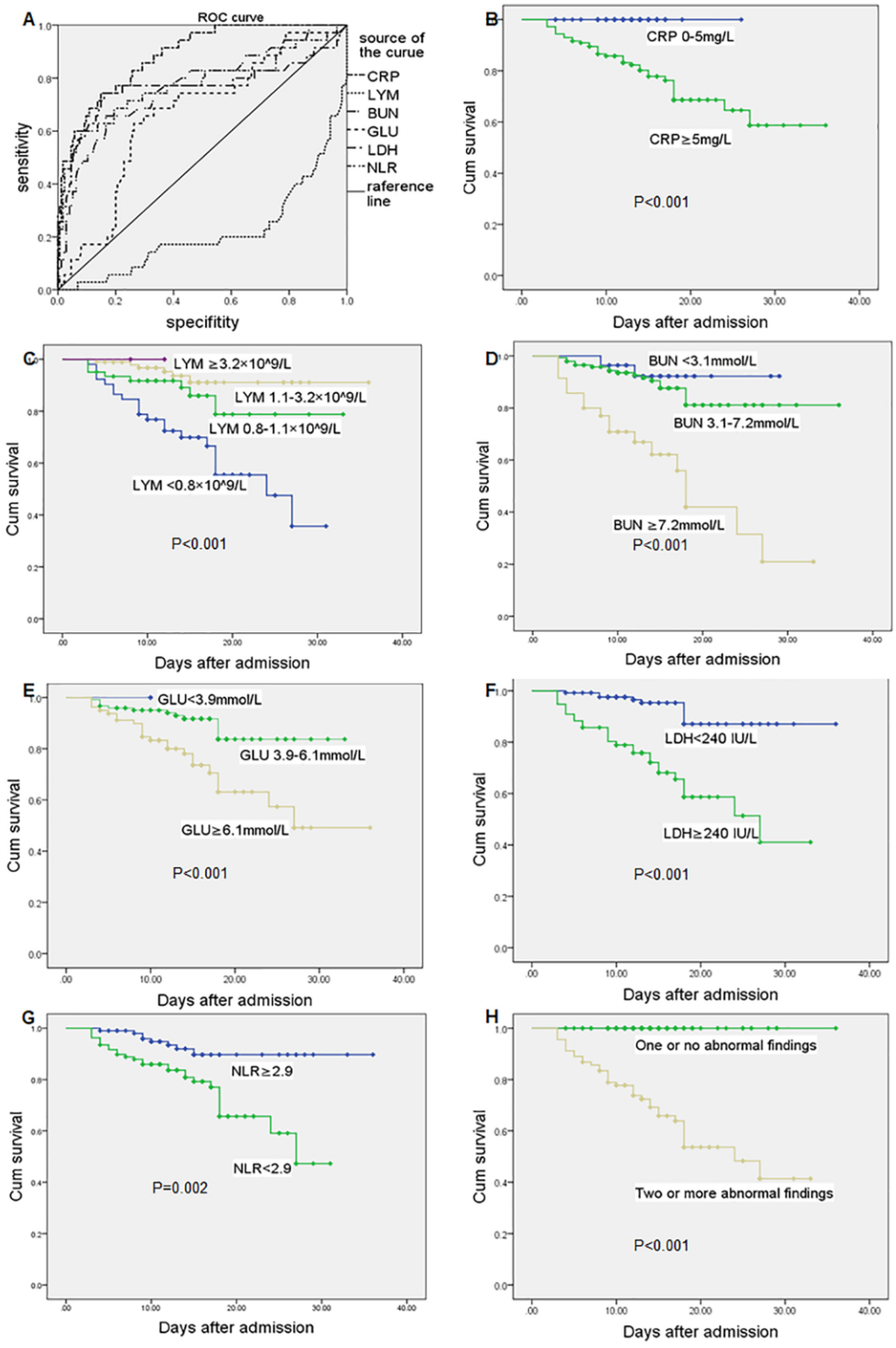

Figure 1. Receiver operating characteristic curve and survival curve. (**A**) ROC in CRP, LYM, BUN, GLU, LDH, NLR at admission. Survival curves in elderly COVID-19 patients with different levels of CRP (**B**), LYM(**C**), BUN (**D**), GLU (**E**), LDH (**F**), NLR (**G**, NLR value take median value in total patients) at admission. (**H**) Two or more abnormal values of CRP, LYM, BUN, LDH in the patients at admission can significantly predict poor prognosis of COVID-19 infected elderly patients. Abbreviations: COVID-19, coronavirus disease 2019; ROC, receiver operating curve; CRP, C-reactive protein; LYM, lymphocytes; BUN, blood urea nitrogen; GLU, glucose; LDH, lactate dehydrogenase; NLR, neutrophil-to-lymphocyte ratio. P-value reported in each subplot indicates the difference between survival curves by Kaplan-Meier method with log-rank test. P < 0.05 was considered statistically significant.

Table 3. Areas under the curve (AUC) of CRP, LYM, BUN, GLU, LDH, and NLR.

Test result variable (s)	AUC	highest specificity	highest sensitivity	optimal cut-off values
CRP	0.857	0.85	0.74	63 mg/L
LYM	0.214			
BUN	0.769	0.82	0.66	6.1 mmol/L
GLU	0.660	0.76	0.63	6.5 mmol/L
LDH	0.766	0.79	0.74	265 IU/L
NLR	0.774	0.69	0.60	6.48

Abbreviations: AUC, areas under the curve; BUN, blood urea nitrogen; CRP, C-reactive protein; GLU, glucose; LYM, lymphocytes; NLR, neutrophil-to-lymphocyte ratio.

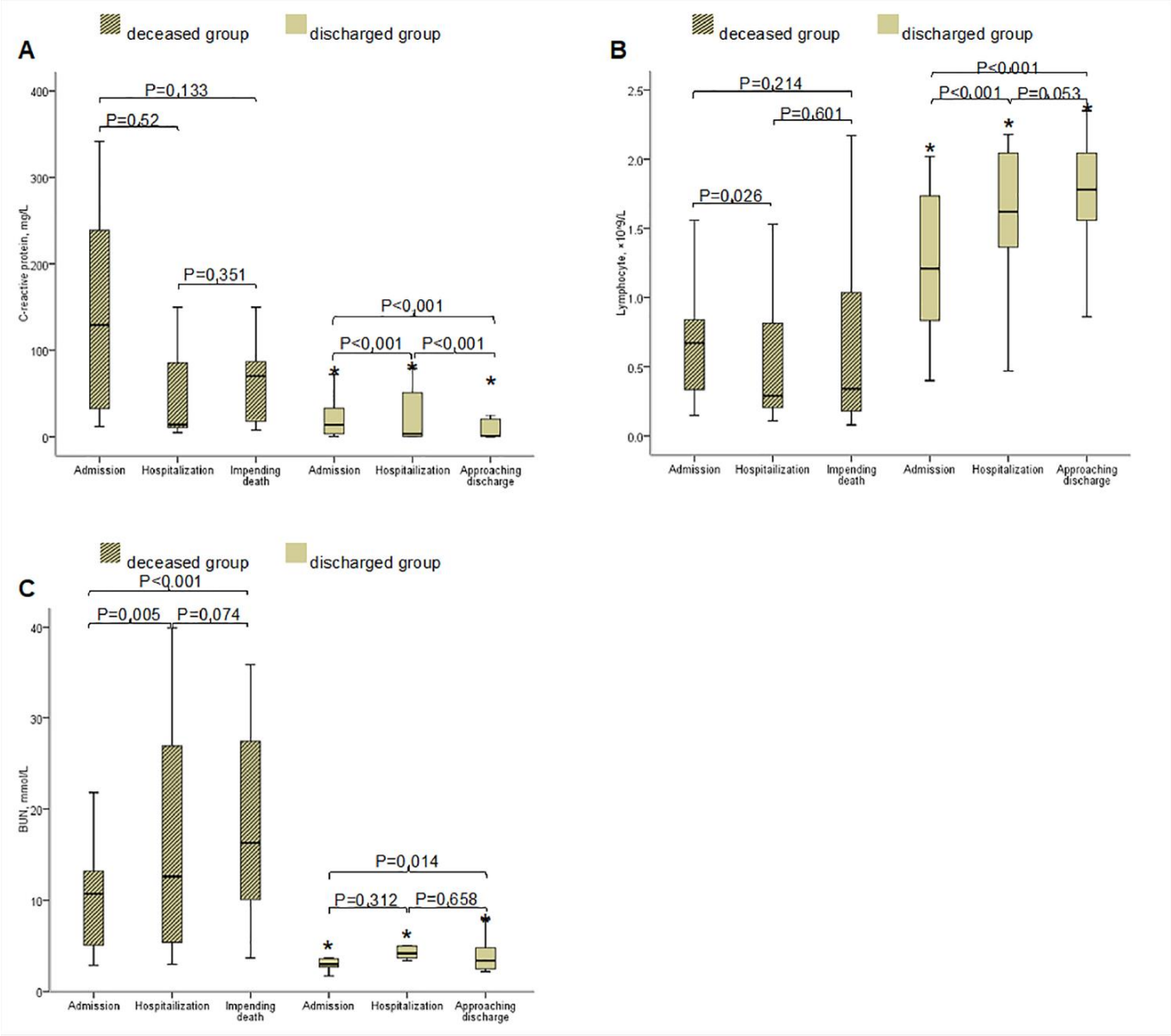

Figure 2. Dynamic Changes of C-reactive protein (**A**), lymphocyte (**B**) and BUN (**C**) within 24 hours at admission, during hospitalization and before discharge or death. Abbreviations: BUN, blood urea nitrogen. The horizontal lines represent the median value in each group. P values indicate differences among admission, hospitalization, impending death between the discharged group and the deceased group. *P<0.05 vs. deceased group. P<0.05 was considered statistically significant.

lack of adequate information or experience regarding what are the most fatal risk factors for the elderly patients, in addition to the generally known risk factors such as comorbidities. The higher mortality in the elderly patients could be in part due to the hypo-immunity, as less robust immune responses in elderly patients may render them more susceptible to ARDS after SARS-CoV-2 infection and die from respiratory failure [10]. Indeed, in the present study, significantly more patients in the deceased group suffered from lymphopenia and failed to survive ICU and wean from mechanical ventilation, while those who survived usually had relatively normal lymphocyte level or lymphocyte levels could gradually recover.

A study showed that male patients accounted for 67% of critically ill patients in the general population [11]. However, our study did not identify significant gender difference between the deceased and discharged elderly. This is possibly because that female patients in our study are at postmenopausal age. SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as a functional receptor [12, 13] and infects type 2 alveolar epithelial cells, which subsequently generates strong immune response and even induces cytokine storm [14]. In our study, the lymphocytes in the deceased group decreased progressively while neutrophils increased, leading to most significantly increased NLR, which is predictive of mortality. Another manifestation of cytokine storm is the elevation of C-reactive protein. In our study, the lever of C-reactive protein in the deceased group was significantly higher when compared with the discharged group. And, the receiver operating characteristic curve analysis indicates that high level of C-reactive protein is a risk factor of mortality in elderly patients. The fact that the levels of C-reactive protein significantly decreased after treatment in the discharged group but not in the deceased group (Figure 2A) provides support that the dynamic changes of C-reactive protein may serve as good indicator of prognosis of the elderly patients with COVID-19. Also, recent study showed that SARS-CoV-2 may directly affect kidney cells [15] and the myocardium [16], these may explain why high BUN and LDH are also highly predictive of mortality in the elderly, despite that LDH is a non-specific myocardial injury marker.

Limitations

This study has several limitations. Firstly, it is a single-center, retrospective study, and included participants were elderly patients who aged over 65 years, therefore, it is limited in sample size. Secondly, elderly patients are special, especially patients with older age, may cause recall bias when conducting epidemiological investigations, especially if there are comorbidities that are used as an analysis of prognosis-related factors.

CONCLUSIONS

This study shows that elderly patients with comorbidities had a greater risk of death, and, the enhanced level of C-reactive protein, blood urea nitrogen or lactate dehydrogenase at admission, progressively lowered lymphocyte counts during hospitalization, alone and especially in combination predict the poor prognosis in elderly patients with COVID-19.

MATERIALS AND METHODS

Study population

This study was a single-center, retrospective, observational study. We included elderly patients aged ≥65 years who were admitted to Wuhan Third Hospital, Wuhan, China, one of the designated hospitals for the treatment of COVID-19 assigned by the government, during the period from January 23, 2020 to February 29, 2020. For all patients, the ethics committee of Wuhan Third Hospital approved this study (Wu San Yi Lun KY2020-019) and granted a waiver of informed consent from study participants.

We included patients who were confirmed with COVID-19 according to World Health Organization interim guidance [17], and laboratory confirmation of SAR-CoV-2 was done by quantitative RT-PCR on samples from the respiratory tract, which was performed by the local health authority. Discharge criteria for patients include: body temperature returned to normal for more than 3 days; the respiratory symptoms had improved significantly, the pulmonary imaging showed a significant improvement of acute exudative lesions, and the nucleic acid test result of respiratory specimens of sputum and/or nasopharyngeal swabs became negative for two successive times (sampling interval more than 24 hours). Patients who were transferred to Huoshenshan Hospital and Leishenshan Hospital during the disease progress and thus the records were not complete at the Wuhan Third Hospital, and patients who were only subjected to one laboratory test during their admission were excluded. The included patients were divided into the discharged group and the deceased group according to the prognosis of patients.

Data collection

A trained team of physicians and medical staffs reviewed and collected epidemiological, demographic, clinical, and prognosis data from electronic medical records, and the records were double checked and confirmed by two researchers (SG and WJ) respectively. The recorded comorbidities included hypertension, diabetes, cardiovascular diseases, chronic

obstructive pulmonary diseases (COPD), respiratory diseases, cerebrovascular diseases, chronic liver disease, digestive diseases, chronic kidney disease and malignancy. The signs and symptoms including fever, cough, headache, fatigue, nausea or vomiting, anorexia, myalgia, chest stuffiness, dyspnea, and diarrhea were recorded. The patients' life vital signs including heart rate, respiratory rate, and mean arterial pressure (MAP) were also collected.

The laboratory findings were collected within 24 hours on admission, which included leucocytes, C-reactive protein, lymphocytes, neutrophils, NLT, ALT, AST, Cr, BUN, CK, CK-MB, coagulation function, fasting blood glucose, albumin, total bilirubin, triglycerides, total cholesterol, and LDH. Lymphopenia was diagnosed as the counts of lymphocytes below 0.8×10^9/L according to the Common Terminology Criteria for Adverse Events version 5.0 [18]. Hyperglycemia was defined as concentrations of fasting blood glucose above 6.1 mmol/L. Hypoalbuminemia was diagnosed as concentrations of albumin below 30 g/L according to American Society of Chest Physicians/ Society of Critical Care Medicine criteria [19].

The treatments included antiviral treatment, antibiotic therapy, glucocorticoid therapy, gamma globulin therapy, albumin therapy, oxygen inhalation, mechanical ventilation, and CRRT. The duration of antiviral therapy was 7-10 days, which included the applications of oseltamivir, ganciclovir, and arbidol. While the antibiotic therapy lasted for 14 days, which included the use of cefoperazone sulbactam and moxifloxacin. No patients received treatments for specific interleukin 6 (IL-6) inhibition or anti-cytokine-storm medications. The complications included ARDS, acute renal failure, and cerebral infarction.

Definitions

The COVID-19 onset time was defined as the date when the first sign or symptom was noticed. Acute cardiac injury was identified if the cardiac biomarkers (e.g. hypersensitive troponin I, Creatine kinase–MB) were above the 99% upper reference limit or new abnormalities were shown in electrocardiography and echocardiography [20]. Respiratory failure was identified according to the guidance of World Health Organization for COVID-19 [17]. Acute kidney injury was defined according to the KDIGO clinical practice guidelines [21]. Cerebral infarction was diagnosed according to the 2018 Stroke Guidelines [22].

Outcomes

The primary outcomes were death and successful discharge of the patients. The second outcomes were laboratory results, radiological data, treatments, and complications of the groups and the analysis of their prognostic values.

Statistical analysis

The categorical variables were compared by chi-square test or Fisher's test, and expressed as frequency and percentage; the continuous variables were compared by rank sum test, and presented as median (IQR) between the discharged group and deceased group. AUC of receiver operating characteristic (ROC) was calculated to predict the prognosis of elderly patients. Survival curve was developed using the Kaplan-Meier method with log-rank test to predict death or discharge in the elderly. A two-sided P value less than 0.05 was considered statistically significant. The SPSS 21.0 software was used for all the analyses.

AUTHOR CONTRIBUTIONS

SG had the idea for the study. JP and ZX designed the study and have full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. WJ, SG, YS, LY, YX, LJ and BW collected the data. SG, FJ and WJ performed data analysis. SG, YC, HL and ZX participated in discussion/data interpretation. SG, FJ and YC drafted the manuscript. ZX and JP revised the final manuscript.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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REFERENCES

1. Epidemiology Working Group for NCIP Epidemic Response and Chinese Center for Disease Control and Prevention. The Epidemiological Characteristics of an Outbreak of 2019 Novel Coronavirus Diseases (COVID-

19) in China. Zhong Hua Liu Xing Bing Xue Za Zhi. 2020; 41:145–51.
https://doi.org/10.3760/cma.j.issn.0254-6450.2020.02.003
PMID:32064853

2. Bajema KL, Oster AM, McGovern OL, Lindstrom S, Stenger MR, Anderson TC, Isenhour C, Clarke KR, Evans ME, Chu VT, Biggs HM, Kirking HL, Gerber SI, et al, and 2019-nCoV Persons Under Investigation Team, and 2019-CoV Persons Under Investigation Team. Persons evaluated for 2019 novel coronavirus - United States, January 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:166–70.
https://doi.org/10.15585/mmwr.mm6906e1
PMID:32053579

3. Olsen SJ, Chen MY, Liu YL, Witschi M, Ardoin A, Calba C, Mathieu P, Masserey V, Maraglino F, Marro S, Penttinen P, Robesyn E, Pukkila J, and European COVID-19 Work Group. Early introduction of severe acute respiratory syndrome coronavirus 2 into europe. Emerg Infect Dis. 2020; 26:1567–70.
https://doi.org/10.3201/eid2607.200359
PMID:32197059

4. Porcheddu R, Serra C, Kelvin D, Kelvin N, Rubino S. Similarity in case fatality rates (CFR) of COVID-19/SARS-COV-2 in Italy and China. J Infect Dev Ctries. 2020; 14:125–28. https://doi.org/10.3855/jidc.12600
PMID:32146445

5. Jung SM, Akhmetzhanov AR, Hayashi K, Linton NM, Yang Y, Yuan B, Kobayashi T, Kinoshita R, Nishiura H. Real-time estimation of the risk of death from novel coronavirus (COVID-19) infection: inference using exported cases. J Clin Med. 2020; 9:523.
https://doi.org/10.3390/jcm9020523
PMID:32075152

6. CDC COVID-19 Response Team. Severe outcomes among patients with coronavirus disease 2019 (COVID-19) - United States, February 12-March 16, 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:343–46.
https://doi.org/10.15585/mmwr.mm6912e2
PMID:32214079

7. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72 314 cases from the chinese center for disease control and prevention. JAMA. 2020. [Epub ahead of print].
https://doi.org/10.1001/jama.2020.2648
PMID:32091533

8. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, Xing F, Liu J, Yip CC, Poon RW, Tsoi HW, Lo SK, Chan KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet. 2020; 395:514–23.
https://doi.org/10.1016/S0140-6736(20)30154-9
PMID:31986261

9. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Zhou X, Du C, Zhang Y, Song J, Wang S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern Med. 2020. [Epub ahead of print].
https://doi.org/10.1001/jamainternmed.2020.0994
PMID:32167524

10. Chen T, Dai Z, Mo P, Li X, Ma Z, Song S, Chen X, Luo M, Liang K, Gao S, Zhang Y, Deng L, Xiong Y. Clinical characteristics and outcomes of older patients with coronavirus disease 2019 (COVID-19) in Wuhan, China (2019): a single-centered, retrospective study. J Gerontol A Biol Sci Med Sci. 2020. [Epub ahead of print].
https://doi.org/10.1093/gerona/glaa089
PMID:32279081

11. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81.
https://doi.org/10.1016/S2213-2600(20)30079-5
PMID:32105632

12. Jiang F, Deng L, Zhang L, Cai Y, Cheung CW, Xia Z. Review of the clinical characteristics of coronavirus disease 2019 (COVID-19). J Gen Intern Med. 2020; 35:1545–49.
https://doi.org/10.1007/s11606-020-05762-w
PMID:32133578

13. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020; 181:271–80.e8.
https://doi.org/10.1016/j.cell.2020.02.052
PMID:32142651

14. Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B, Yang P, Sarao R, Wada T, Leong-Poi H, Crackower MA, Fukamizu A, Hui CC, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature. 2005; 436:112–16.
https://doi.org/10.1038/nature03712
PMID:16001071

15. Pan XW, Xu D, Zhang H, Zhou W, Wang LH, Cui XG. Identification of a potential mechanism of acute kidney injury during the COVID-19 outbreak: a study based on single-cell transcriptome analysis. Intensive Care Med.

2020; 46:1114–16.
https://doi.org/10.1007/s00134-020-06026-1
PMID:32236644

16. Tavazzi G, Pellegrini C, Maurelli M, Belliato M, Sciutti F, Bottazzi A, Sepe PA, Resasco T, Camporotondo R, Bruno R, Baldanti F, Paolucci S, Pelenghi S, et al. Myocardial localization of coronavirus in COVID-19 cardiogenic shock. Eur J Heart Fail. 2020; 22:911–15.
https://doi.org/10.1002/ejhf.1828
PMID:32275347

17. World Health Organization. Clinical management of severe acute respiratory infection when novel coronavirus (nCoV) infection is suspected: interim guidance. Published January 28, 2020. https://www.who.int/publications-detail/clinical-management-of-severe-acute-respiratory-infection-when-novel-coronavirus-(ncov)-infection-is-suspected.

18. National Cancer Institute. Common Terminology Criteria for Adverse Events (CTCAE) [Internet]. 2018 [cited 2019 Aug 5]. https://ctep.cancer.gov/protocoldevelopment/electronicapplications/ctc.htm#ctc_50

19. Calandra T, Cohen J, and International Sepsis Forum Definition of Infection in the ICU Consensus Conference. The international sepsis forum consensus conference on definitions of infection in the intensive care unit. Crit Care Med. 2005; 33:1538–48.
https://doi.org/10.1097/01.ccm.0000168253.91200.83
PMID:16003060

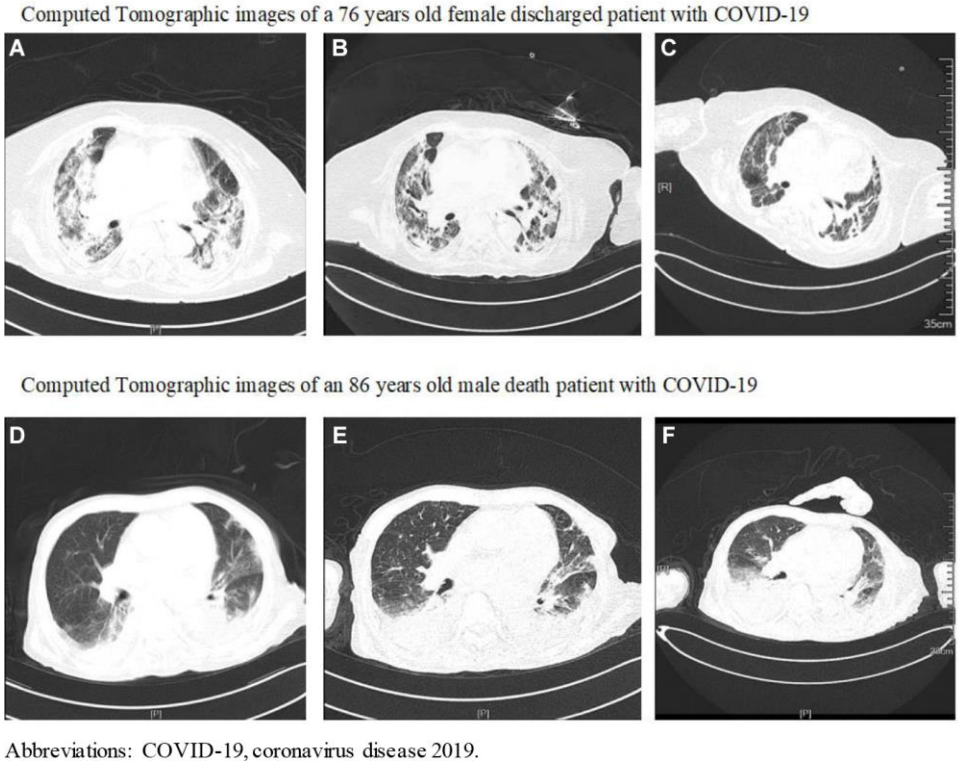
20. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506.
https://doi.org/10.1016/S0140-6736(20)30183-5
PMID:31986264

21. Kellum JA, Lameire N, and KDIGO AKI Guideline Work Group. Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (part 1). Crit Care. 2013; 17:204.
https://doi.org/10.1186/cc11454 PMID:23394211

22. McCoy CE, Langdorf MI, Lotfipour S. American heart association/american stroke association deletes sections from 2018 stroke guidelines. West J Emerg Med. 2018; 19:947–51.
https://doi.org/10.5811/westjem.2018.9.39659
PMID:30429926

SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Computed Tomographic (CT) findings of two patients. As shown in (**A**–**C**) a 76 years old female discharged patient, she had fever and headache for 5 days before admission on January 28, 2020. (**A**) image obtained on day 23 after symptom onset shows progressive multiple ground glass opacities, massive high-density shadows in bilateral lungs. (**B**) image obtained on day 28 after symptom onset shows multiple ground glass opacities and high-density shadows in bilateral lungs. (**C**) image obtained on day 34 after symptom onset showed that the consolidation was obviously resolved and pulmonary interstitial fibrosis attenuated. The patient was discharged on March 17, 2020, the duration from admission to discharge was 49 days, and the duration from onset of symptoms to discharge was 54 days. (**D**–**F**) an 86 years old male death patient, he had cough and chest tightness for 7 days before admission on February 25, 2020. (**D**) image obtained on day 8 after symptom onset showed multiple ground glass opacities, high-density shadows in bilateral lungs. (**E**) image obtained on day 14 after symptom onset showed progressive multiple ground glass opacities and mass shadows of high-density shadows in bilateral lungs. (**F**) image obtained on day 21 after symptom showed progressive multiple ground glass opacities and mass shadows of high-density shadows in bilateral lungs. The patient died on March 10, 2020, and the duration from admission to death was 14 days, while the duration from onset of symptoms to death was 21 days.

Review

Pathophysiology of SARS-CoV-2 infection in patients with intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) is associated with old age and underlying conditions such as hypertension and diabetes. ICH patients are vulnerable to SARS-CoV-2 infection and develop serious complications as a result of infection. The pathophysiology of ICH patients with SARS-CoV-2 infection includes viral invasion, dysfunction of the ACE2–Ang (1–7)–MasR and ACE–Ang II–AT_1R axes, overactive immune response, cytokine storm, and excessive oxidative stress. These patients have high morbidity and mortality due to hyaline membrane formation, respiratory failure, neurologic deficits, and multiple organ failure.

INTRODUCTION

Novel coronavirus (2019-nCoV)–associated pneumonia cases first appeared in Wuhan, Hubei Province, China, in December 2019 [1]. Whole-genome sequencing identified a novel coronavirus—severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2, 3]. In the following months, SARS-CoV-2 rapidly spread throughout China and the world. By May 26, 2020, SARS-CoV-2 had resulted in 84,543 infections and 4,645 deaths in China, as reported by National Health Commission of the People's Republic of China. In addition, other countries reported 5,468,627 confirmed cases and 345,544 deaths. The World Health Organization declared SARS-CoV-2 a public health emergency and named the virus Corona Virus Disease 2019 (COVID-19).

Although the source of SARS-CoV-2 and its pathogenesis are still being studied, COVID-19 is a systemic disease that can lead to pneumonia, respiratory failure, and acute respiratory distress syndrome (ARDS) and has high morbidity and mortality. COVID-19 also affects the cardiovascular, renal, cerebrovascular, and blood coagulation systems. Genome sequencing of patients' cerebrospinal fluid has identified the presence of SARS-CoV-2 in the brain, which is also seen in SARS and Middle East respiratory syndrome (MERS) infection [4]. Here, we review the pathophysiology of SARS-CoV-2 infection in patients with intracerebral hemorrhage (ICH).

Characteristics of SARS-CoV-2

Coronaviruses (CoVs), part of the subfamily *Orthocoronavirinae* in the family *Coronaviridae* of the order Nidovirales, are enveloped, nonsegmented, positive-sense, single-stranded RNA viruses [5]. Some CoVs are transmitted from animals to people and have gradually developed as pathogens of the respiratory, gastrointestinal, and central nervous systems in human. Examples include SARS, which caused an outbreak in 2002, and MERS, which caused an outbreak in 2012, both of which affect the lower respiratory tract [6, 7]. Genome sequencing has identified 2019-nCoV as a *Betacoronavirus*. SARS-CoV-2 and two bat-derived SARS-like strains, ZC45 and ZXC21, form an independent clade within lineage B of the subgenus *Sarbecovirus* [8, 9]. The two bat SARS-related

coronaviruses closest to SARS-CoV-2, ZXC21 and ZC45, can infect suckling rats and cause brain tissue inflammation and pathological changes in the lung and intestine [10].

SARS-CoV-2 contains a single positive-sense RNA genome and is around 60 to 140 nm in diameter [5]. The genome sequence of SARS-CoV-2 has 89% nucleotide identity with the bat SARS-like CoV ZXC21, 86.9% with the bat SARS-like CoV ZC45, and 82% with the human SARS-CoV [10–12]. The phylogenetic trees of SARS-CoV-2's orf1a/b, spike, envelope, membrane, and nucleoprotein also cluster closely with those of the bat, civet, and human SARS coronaviruses [10, 11, 13]. However, the external subdomain of spike's receptor binding domain in SARS-CoV-2 shares only 40% amino acid identity with other SARS-related coronaviruses [8, 10].

SARS-CoV-2, like SARS-CoV, manipulates angiotensin-converting enzyme 2 (ACE2) as the viral receptor and invades type 2 alveolar epithelial cells in the lower respiratory tract [11]. ACE2 inhibitors prevent SARS coronavirus from constant viral replication in Vero E6 cells [14]. The receptor binding domain on the S1 subunit of the SARS-CoV-2 spike protein (S glycoprotein) and the transmembrane domain of ACE2 are implicated in SARS-CoV-2 infection [2, 15].

A majority of the earliest confirmed patients infected with SARS-CoV-2 were exposed to wild animals sold in the Huanan Seafood Wholesale Market. Although it is difficult to pinpoint the exact source or the intermediate host of the novel coronavirus, the first cluster of pneumonia cases suggests that person-to-person transmission via the respiratory route occurred [16]. The digestive system is also hypothesized to be a route of SARS-CoV-2 transmission.

ICH patients are vulnerable to SARS-CoV-2 infection and develop serious complications as a result of infection

The general population is susceptible to SARS-CoV-2 infection. As of February 11, 2020, the Chinese Center for Disease Control and Prevention had identified 72,314 cases of COVID-19, including 55,239 confirmed patients, 16,186 suspected infections, and 889 infections without any symptoms [17]. 87% of the patients are between 30 and 79 years old. Clinical symptoms at the beginning of COVID-19 infection include chills, fever, cough, fatigue, myalgia, dyspnea, and diarrhea. Chest computed tomography (CT) images show ground-glass opacity in both lungs and, in severe cases, progressive consolidation of multiple lobular and subsegmental tracts. However, many infected patients are asymptomatic and have normal chest CT scans. Asymptomatic patients with SARS-CoV-2 infection, as well as those with atypical neurologic manifestations such as headache, dizziness, nausea, and vomiting, contribute to misdiagnosis and delayed treatment. According to the Chinese Center for Disease Control and Prevention, 81% of the 72,341 patients diagnosed with COVID-19 had mild disease, and the mortality rate was approximately 2.3%. However, the fatality rate increased to 8.0% in people age 70 to 79 years old and 14.8% in those age 80 or older. Infected patients with underlying diseases also had higher fatality rates: 10.5% in patients with cardiovascular disease, 7.3% for diabetes, 6.0% for hypertension, and 5.6% for cancer. A review of the clinical features of 138 confirmed patients in Zhongnan Hospital of Wuhan University confirmed that ICU patients were obviously elder and were more likely to have underlying diseases, as well as having higher risk for poor outcome [18]. Therefore, the worst complications and outcomes occur in older patients and those with chronic diseases, such as pulmonary disease, diabetes, hypertension, heart failure, atherosclerosis, cerebrovascular disease, and cancer. Patients with severe SARS-CoV-2 infection develop pneumonia and extrapulmonary pathological changes. Complications in patients with severe infection include hypoxemia, pulmonary edema, ARDS, postviral bacterial superinfection, septic shock, metabolic acidosis, blood coagulation dysfunction, and multiple organ damage. A retrospective, single-center study of 99 cases of COVID-19 in Wuhan Jinyintan Hospital revealed that severe patients had high levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), myocardial zymogram, blood urea nitrogen and serum creatinine, all of which were implicated with multiple organ damage. Biopsy samples of tissues from patients with SARS-CoV-2 indicate impairment of alveolar epithelial cells and pneumocytes in both lungs, exudation of extracellular fluid in alveolus, infiltration of lymphocytes and macrophages, and formation of hyaline membrane, indicating ARDS, which also occurs in SARS and MERS coronavirus infection [19, 20].

ICH accounts for 20% to 30% of strokes in China and is associated with high mortality and morbidity, with most survivors experiencing neurologic and cognitive impairment. The physiological status go to the bad with age and elder persons have higher possibility to develop underlying diseases, consisting of hypertension, diabetes, and dysfunction of blood coagulation, all of which are interact with the occurrence and development of ICH [21]. Hypertension is the mainly risk factor of ICH, as well as amyloid angiopathy, hemangioma, arteriovenous malformations, coagulopathy, and cerebroma [22]. Therefore, ICH is associated with old age and underlying conditions such as hypertension and

diabetes. ICH patients, susceptible to SARS-CoV-2 infection, are prone to develop serious complications and need ICU admission.

ICH exerts mass effect and causes primary physical damage that is dependent on the location, volume, and expansion of the hematoma. Secondary injury is caused by brain edema, the inflammatory cascade, and hematoma decomposition products. After the interaction between SARS-CoV-2 and the ACE2 receptor, some infected patients rapidly develop elevated blood pressure, which brings about severe cerebral changes, including activated microglia, accumulated ferritin, damaged neurons, and impaired neurologic function [23]. One report describing 41 cases of COVID-19 indicated that prolonged prothrombin time, elevated D-dimer, and severe platelet reduction occur in ICU patients with SARS-CoV-2 infection [24]. Then ICH patients may develop blood coagulation dysfunction as a result of infection. The high levels of thrombin is a trigger of early perihematomal brain edema; thrombin affects a variety of cells, including microglia, neurons, and brain endothelial cells, and destroys the blood–brain barrier (BBB) [25]. Low platelet activity is a marker of severe ICH, and platelet transfusion in the acute phase can limit hemorrhage volume and attenuate poor outcomes [26]. The BBB inhibits cerebrum invasion, regulates substantial exchange, and maintains homeostasis in the center nervous system. The viral invasion and breakdown of the BBB results in immunocyte recruitment in the central nervous system. Overactivation of the immune response and pro-inflammatory factors can lead to cellular apoptosis and necrosis, endothelial impairment, brain edema, and neuronal loss. In detail, the pathophysiology of ICH patients with SARS-CoV-2 infection includes viral invasion, dysfunction of the ACE2–Ang (1–7)–MasR and ACE–Ang II–AT_1R axes, overactive immune response, cytokine storm, and excessive oxidative stress.

SARS-CoV-2 brain invasion and ACE2

The renin-angiotensin system (RAS) consists of the protease renin, angiotensinogen, angiotensin-converting enzyme (ACE), and angiotensin II. The local brain RAS includes angiotensinogen, peptidases, angiotensins, and specific receptor proteins that play specific roles in development of cerebrovascular disease [27, 28]. ACE2, a homologous enzyme of ACE, is secreted by endothelia and smooth muscle cells. A study pointed that SARS-CoV-2 can manipulate all but mouse ACE2 as the entry receptor in the ACE2-expressing cells, which might permit the viral invasion and replication in multiple organs. ACE2 is found in arterial and venous endothelial cells and arterial smooth muscle cells in most organs, including oral and nasal mucosa, naso-pharynx, lung, stomach, small intestine, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, kidney, and brain [14, 29].

Pathologists obtained human brain tissue from autopsies and research on the staining for ACE2; endothelial and smooth muscle cells of cerebrum were stained [14]. The barrier between plasma and brain cells is formed by brain capillary walls and glial cells and the barrier between plasma and cerebrospinal fluid is formed by choroid plexus. The expression of ACE2 in endothelial and smooth muscle cells allow viral invasion and replication in the blood-brain barrier. The BBB breakdown includes swelling of endothelial cells, necrosis, apoptosis, inflammatory injury and systemic vasculitis. Genome sequencing of patients' cerebro-spinal fluid confirmed SARS-CoV-2 infection in the brain [17]. The infected patients with atypical neurologic manifestations such as headache, dizziness, nausea, and vomiting are important signs for SARS-CoV-2 brain invasion. In addition, autopsies from patients with SARS infection have detected SARS-CoV particles and genomic sequence in cerebral neurons, as well as in T lymphocytes and monocytes in the circulating blood of multiple organs [30]. After intranasal inoculation of MERS-CoV in transgenic mice, study of brain tissues indicated viral invasion. Mice infected with the JHM and A59 strains of murine hepatitis virus (MHV) manifest an acute encephalo-myelitis and gradually develop demyelinating disease as a result of persistent viral stimulation. In addition to pulmonary disease, coronaviruses also cause patho-logical changes in the cerebrum due to their neuroinvasive and neurotropic properties [31].

SARS-CoV-2 and the dysfunction of the ACE2–Ang (1–7)–MasR and ACE–Ang II–AT_1R axes

Angiotensin 1-7, which is transferred by endopeptidases, ACE2, and ACE from angiotensin I, binds to the Mas receptor and is an effective and protective vasodilator [32]. Mas receptors are distributed throughout the brain, including the medulla and forebrain, which are associated with cardiovascular regulation, and the hippocampus, amygdala, anterodorsal thalamic nucleus, cortex, and hypoglossal nucleus [33]. In contrast to the effects of Ang II in the brain, Ang-(1-7) regulates the cardiovascular reflex and mediates blood pressure by releasing nitric oxide (NO) and activating the PI3K-Akt-PKB pathway [34]. The interaction between Ang-(1-7) and the Mas receptor decreases reactive oxygen species (ROS) production by cleaving Ang II or inhibiting AT_1 receptors [35]. Ang-(1-7) and the G-protein-coupled receptor Mas, which initiate the release of cytokines and activate and recruit leukomonocytes, reduce inflammation by the restraining Des-Arg9 bradykinin (DABK)-mediated pathway [36, 37]. The ACE2–Ang-(1-7)–Mas axis is a

protective regulator in the center nervous system; it regulates blood pressure and inhibits inflammatory injury, oxidative stress, fibrosis, and cellular apoptosis [38, 39]. Injection of Ang-(1-7) in the ventricle of rats reduces ICH-induced injury, resulting in limited hematoma expansion, decreased microglia, and neuronal recovery [40]. In addition, administration of Ang-(1-7) in mice with aneurysmal rupture inhibits the production of TNF-α and IL-1β and attenuates pathological damage [41]. The ACE2–Ang (1–7)–MasR axis and ACE–Ang II–AT_1R axis counterbalance each other to maintain cerebral homeostasis [42]. Thus, pathological disruption of ACE2 and Ang II can result in neurologic damage [43].

Infection and endocytosis of SARS-CoV-2 particles downregulate active ACE2 and Ang-(1-7) and increase Ang II. The subsequent inhibition of the ACE2–Ang (1–7)–MasR axis and overactivation of the ACE–Ang II–AT_1R axis underlie the progressive pathological deterioration in the cerebrum seen in patients with SARS-CoV-2. Disruption of the ACE–Ang II–AT_1R axis contributes to rapidly elevated blood pressure [44]. Stimulation and production of Ang II in local brain, which binds to AT_1 receptors, activates the inflammatory NF-κB pathway and superoxide production by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [45]. Increased ROS production damages brain tissue, which is full of polyunsaturated fatty acid. In addition, overactivation of ACE–Ang II–AT_1R is partly responsible for brain inflammation and cellular apoptosis and necrosis, leading to endothelial impairment, brain edema, and neuronal injury. Administration of brainc Ang II receptor inhibitor attenuated acute inflammatory responses in an animal model with bacterial infection [46]. The brain inflammation with positive feedback seen in ICH patients with SARS-CoV-2 infection, which is postulated to be a result of dysfunction of the ACE2–Ang (1–7)–MasR and ACE–Ang II–AT_1R axes, results in excessive oxidative stress and elevated cytokines, chemokines, and toxic substances, which lead to neuronal injury, cell death, brain edema, and neurologic deficits. Hematoma expansion and brain edema contribute to physical pressure on neighboring structures, such as arterial vessels, the aqueduct of Sylvius, and the brainstem, leading to cerebral ischemia, obstructive hydrocephalus, cardiorespiratory dysfunction, intracranial hypertension, and even cerebral hernia.

SARS-CoV-2, immune evasion and over-activated immune responses

Among hospitalized patients with SARS-CoV-2 infection, general laboratory abnormalities include leukopenia and lymphopenia. These abnormalities indicate that both the viral burden and the reaction of immune system play a critical role in SARS-CoV-2 invasion and replication. The immune system can inhibit coronavirus, clean up apoptotic cells, and promote tissue recovery in the cerebrum. Chemotactic factors help leukocytes migrate to the correct position to fight infection, and abnormal secretion can aggravate the cerebral immunopathology. Conversely, weak immune systems and insufficient immune responses are associated with viral survivors and rapid coronavirus invasion. Therefore, the relationship between SARS-CoV-2 infection and the immune response needs to be investigated, with potential measures provided to interfere with viral dissemination, clear the virus, and reduce tissue impairment.

After the internalization of coronavirus particles, host cells recognize the coronavirus and initiate an innate and adaptive immune response against the viral infection; the complement system is also activated. Interaction between cell-surface pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs), activation of proinflammatory signaling proteins and pathways, production and release of several inflammatory factors, and migration of immunocytes occur in the immune and inflammatory settings [36]. In addition, complementary autocrine and paracrine signaling ensures that the infected cells and surrounding uninfected cells express a series of interferon-stimulated genes (ISGs), which establish an antiviral microenvironment [47]. The PRRs in host cells that detect pathogens contain toll-like receptor (TLR), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), C-type lectin-like receptor (CLR), cytoplasmic DNA receptor (CDR), type I interferons (IFNs), and dendritic cells (DCs) and restrict viral pervasion with the help of macrophages, natural killer cells, T/B cells, and immune molecules [48]. The main function of macrophages is to phagocytose and digest cell debris and pathogens and activate lymphocytes or other immune cells in response to pathogens. Macrophages and DCs infected with feline infectious peritonitis virus (FIPV) inhibited the protective Th1 cell response by promoting the signaling pathway of IL-10 expression [49]. Natural killer cells are active in the response to numerous infectious diseases and regulate immune response by activating a series of cytokines including IL-12, IL-1β, IL-18, IL-23, and IFN-β, sometimes resulting in hypersensitivity reactions and autoimmune diseases [50]. B cell, $CD4^+$ T cells, and $CD8^+$ T cells, with migration and secretion features, exert important protective functions during adaptive immune responses in organisms. $CD4^+$ helper T cells fight pathogens by activating T-cell-dependent B cells and supporting humoral and cellular immunity. Cytotoxic $CD8^+$ T cells kill infected cells using a specific antigen response that corresponds with tissue damage [51]. Due to the antigenic stimulation and activation of antigen-

presenting cells and Th cells, activated B cells differentiate into plasma cells and secrete pathogen-specific antibodies to inhibit the effects of pathogens.

Although SARS-CoV-2 is sensitive to cell-surface PRRs, immune evasion is achieved by defending intermediate products of viral replication from immune recognition, resulting in spread of SARS-CoV-2 and restricted immune responses, which are associated with lymphopenia [47, 52]. However, although immune evasion of SARS-CoV-2 temporarily restricts the innate immune response, subsequent overactivation or eruptive initiation of the immune system can occur, leading to multiple organ damage [53]. A hyperactivated immune response contributes to immunopathogenesis, tissue damage, and severe complications. The presence of lymphopenia in 2019-nCoV infection indicates that SARS-CoV-2 affects lymphocytes. Although the $CD4^+$ and $CD8^+$ T cell levels in peripheral blood are largely decreased, the function of lymphocytes is overactivated. Flow cytometric analysis has indicated high levels of proinflammatory $CCR4^+CCR6^+$ Th17 in $CD4^+$ T cells and cytotoxic granules in $CD8^+$ T cells, which are associated with systemic inflammatory responses and toxic reactions [54, 55]. In addition, the depressed immune response also indicates the mechanism of immune evasion in SARS-CoV infection [56]. $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes were shown to be decreased in the acute phase of SARS-CoV infection, indicating lymphocyte deterioration and a suppressed immune system. Nine hours after the cellular infection of SARS-CoV in vitro, the incomplete viral replication of SARS-CoV led to low production of antiviral cytokines (IFN-α, IFN-β, IFN-γ, and IL-12p40), mild generation of proinflammatory cytokines (TNF-α and IL-6), and significantly elevated inflammatory chemokines (MIP-1a, IP-10, and MCP-1) [57].

Activation of the immune system in response SARS-CoV-2 and subsequent signaling cascades lead to innate and adaptive immunity and proliferation of proinflammatory cytokines, neutralizing antibodies, and recruited lymphocytes, such as neutrophils and macrophages. However, surviving virus excessively stimulates immune cells with positive feedback and causes an inflammatory factor storm. A recent report of 138 patients with SARS-CoV-2 at Zhongnan Hospital of Wuhan University indicated that adverse reactions in severe cases included neutrophilia, coagulation activation, and acute multiple organ injury and that these reactions were associated with higher concentrations of white blood cells and neutrophils, D-dimer, creatine, aspartate aminotransferase, and high-sensitivity troponin I [18]. Another report from Wuhan demonstrated that, compared with healthy people, 44 patients with SARS-CoV-2 had higher immune cytokine counts, including IL-1b, IL-6, IL-12, IFN-γ, IP10, and MCP-1, resulting in systematic toxic organ changes and severe tissue damage [24]. Moreover, patients admitted to the ICU presented with higher levels of GCSF, IP10, MCP-1, MIP1A, and TNF-α [58].

It is believed that the immune response that aims to kill SARS-CoV-2 also disrupts tissue homeostasis and induces immunopathological changes, which is similar to MERS-CoV and SARS-CoV infection [49]. In an analysis of 128 serum samples of SARS patients, T cell responses, especially $CD8^+$ T cell responses, and antibody production were found to be major components of the immune response to SARS-CoV infection. The serological manifestation of memory phenotype ($CD27^+/CD45RO^+$) $CD4^+$ T cells producing IFN-γ, TNF-α, and IL-2 and $CD8^+$ T cells producing IFN-γ, TNF-α, and CD107a was correlated with severe disease. High concentrations of plasma IFN-γ, IL-1β, IL-6, IL-8, IL-12, IP-10, MCP-1, CXCL8, CXCL10, and CCL2 granules are a result of hyperactivated inflammatory signaling cascades and cytokine storm and are associated with the immunopathological changes and severity of SARS-CoV infection [59]. In SARS-CoV infection, neutrophils and chemokines such as IL-8 infiltrate the respiratory tract and generate myeloperoxidase and elastase, which causes deterioration of pulmonary tissue and function and leads to ARDS, respiratory failure, and admission to the ICU. A research investigated 27 serum samples of MERS-CoV from patients from South Korea in 2015 [60]. They found that the $CD8^+$ T cell response and proinflammatory factors are associated with severe disease, whereas $CD4^+$ T cell response is associated with less severe disease. $CD8^+$ T cells act on viral S protein in the early phase of MERS-CoV infection, whereas $CD4^+$ T cells interact with E/M/N proteins in the later phase. The invasion of MERS-CoV in host cells triggers the Th1 and Th17 proinflammatory response and stimulates monocytes and lymphocytes, resulting in high levels of IFN-γ, TNF-α, IL-15, and IL-17 and promoting activation of the MAPK, STAT3, and NF-κB signaling pathways. The downstream signaling protein and secreted inflammatory factors fight against the virus, even leading to tissue damage, via the production of IL-6, IL-1β, TGF-β, TNF-α, IL-8, and MCP-1. In addition, an elevated IL-10 level correlates with activated JAK-STAT pathway and indicates an anti-inflammatory effect [61].

Therefore, decreased lymphocytes and the induction of cytokine storm are potent indicators of severe COVID-19 infection. In a study of 228 patients with SARS, patients with severe disease had high levels of IL-6 and reduced concentrations of IL-8 and TGF-β in the acute phase, which correlated with disease severity [62]. The

cytokine profiles caused by excessive immune response lead to ARDS and multiple organ failure, contributing to the mortality of patients with COVID-19 [63]. Plasma exchange can clear inflammatory factors, block cytokine storms, and reduce the damage caused by the inflammatory response.

SARS-CoV-2 and cytokine storms in ICH patients

After mechanical injury by ICH, activated microglia migrate to the position of damage. Although M1 microglia help clear necrotic substances, they also generate inflammatory cytokines and contribute to BBB breakdown and brain edema. Triggered inflammatory cascades, including production of IL-1β, TNF-α, ROS, chemokines, and prostaglandins, damage the BBB [64]. Due to increased BBB permeability, mobilized neutrophils in the perihematomal region generate ROS and release a series of granules, such as collagenase, myeloperoxidase, and elastase. Neutrophils can stimulate nearby microglia, regulate immune response, and exaggerate adverse effects on brain tissue via production of IL-8, IL-6, TNF-α, and IL-1β, resulting in neuronal loss and brain edema. Persistently high neutrophil levels in peripheral blood predict poor prognosis in ICH patients. In addition, neutrophils are important mediators in the recruitment of monocytes [65]. The reactive astrocytes gather around the hematoma and induce MMP-9 [66]. Elevated MMP-9 activity is associated with perihematomal edema, BBB disruption, and neural loss [67]. $CD8^+$ cytotoxic T cells and $CD4^+$ Th cells increase in the perihematomal region and contribute to neuronal apoptosis and endothelial injury. Due to physical damage and BBB impairment, inflammatory cells infiltrate the hematoma, stimulate the production of cytokines and chemotactic factor with active feedback, and initiate cellular apoptosis via NF-κB inflammatory signaling pathways and downstream molecules [68]. Intercellular adhesion molecule-1, IL-1β, TNF-α, chemokines, MMP-9, inducible nitric oxide synthase, free radicals, COX-2, and PLA_2 participate in NF-κB activation. Inflammatory cells contain recruited neutrophils and monocytes and resident microglia and astrocytes. Active cytokines can stimulate the complement system to form the membrane attack complex and generate C3a and C5a, resulting in direct tissue injury and augmented immune response. Infiltration of blood substances affects microcirculation, contributing to hypoxia and producing ROS. Hemoglobin and iron are cytotoxic and cause oxidative and proinflammatory changes that further brain injury, probably in conjunction with oxygen free radicals [69]. Oxidative stress, excitotoxicity, and cellular necrosis and apoptosis result in neuronal injury, brain edema, and cell death [70].

However, there is limited information about the innate immune responses in the center nervous system after SARS-CoV-2 brain invasion in ICH patients. In a lab study, the serum samples of SARS-CoV-2 patients were IgM positive in the early stage of infection and subsequently became IgG positive, indicating a humoral response [11]. Because SARS-CoV-2 invades the brain via ACE2 receptor in ICH patients, viral pathogenicity and replication destroy the blood–brain barrier and induce dynamic immune responses. SARS-CoV-2 infection may disturb the activation and inhibition of related signaling cascades, leading to stimulation of the innate immune system, recruitment of lymphocytes, secretion of toxic substances, and cytokine storm with positive feedback circulation.

Neurologic biopsies of patients with SARS-CoV-2 infection demonstrate congestion, brain edema, and partial neuronal degeneration, similar to the effects seen with SARS and MERS infection. ACE2 receptors are distributed throughout the synaptic membrane of the brain, and center nervous system autopsies of SARS patients demonstrated infiltration of monocytes and lymphocytes in blood vessels, hydrocephalus, demyelination of the nerve myelin sheath, and neuronal degeneration, which is associated with aggravation of the pathological changes seen in ICH patients [19]. After SARS-CoV infection in K18-hACE2 mice, viral particles and antigens were found in the neurons of the brain. Upregulation of cytokines and chemokines contributes to BBB impairment, gliocyte hyperplasia, neuronal damage, and brain edema as a result of cellular oxidative damage, necrosis, and apoptosis [71]. Some patients with severe MERS-CoV infection manifested neurological symptoms, including epilepsy, dystaxia, paralysis, and conscious disturbance. Magnetic resonance imaging performed in hospitalized patients with MERS indicated acute alterations in the white matter and the subcortical areas of the frontal, temporal, and parietal lobes [72]. In MERS, excessive production of proinflammatory cytokines and chemokines leads to rapid increases of RIG-I, MDA5, PKR, MYD88, TNF-α, IL-1β, CCL2, CCL5, and CXCL10 in the brain [73]. MHV affects oligodendrocytes and impairs the myelin sheath via immunologic injury. Although MHV-JHM infection in brain tissue initiates an immune response and activates inflammatory signaling cascades to clear the virus, MMP secretion, immunocyte migration, and increased chemokines and cytokines are associated with BBB breakdown and demyelination [49]. Impairment of brain microvascular endothelial cells (BMECs) in vitro by MHV3 infection is a result of downregulation of zona occludens protein 1 (ZO-1), VE-cadherin, and occludin, which leads to elevated BBB permeability [74]. In addition, stimulation and recruitment of

macrophages and/or microglia in the white matter contributes to demyelination in MHV-JHM-infected mice. Although immune responses help clear pathogens, excessively inflammatory signaling cascades, influx of cytokines and chemokines, a large volume of recruited immune cells, and toxic substances in the center nervous system indicate a poor prognosis.

Whereas SARS-CoV-2 invasion and replication in brain cause direct damage, indirect deterioration is associated with the immune response. Immune mediator dysfunction and autoimmune reactions prolong the immune response and exacerbate tissue damage. Neutrophils, natural killer cells, macrophages, and lymphocytes proliferate and produce IL-1α, IL-1β, IL-6, IL-12, TNF-α, IFN-γ, and CXCR2. Migrated neutrophils swallow viral particles; generate a series of antibacterial peptides, proteases, and ROS to kill the virus; and introduce tissue damage. ROS, superoxide anion, and NADPH oxidase cause excessive oxidative stress. Elevated neutrophil-to-lymphocyte ratio (NLR) has been shown to be a marker of severe SARS-CoV-2 infection in the early phase.

An overactivated immune system affects both virus and host cells. As SARS-CoV-2 combines with ACE2 receptors, the immunopathological injury in center nerve system is the result of the explosive cytokine storm [75]. ACE2 is highly expressed in arterial and venous endothelial cells and arterial smooth muscle cells in brain. The impairment and contraction of vascular endothelial cells, due to the out of control inflammatory response, lead to increased permeability of the capillary wall and diffusion of substances from vessels into the interstitial space. Brain tissues with ACE2 receptors are attacked the extreme immune response, eventually leading to neurologic deficits and bad outcomes.

CONCLUSIONS

Patients with COVID-19 who present with neurologic symptoms need early diagnosis, isolation, and treatment. When new neurologic symptoms occur in hospitalized patients, such as ataxia, focal motor deficits, and conscious disturbance, cerebrospinal fluid examination and SARS-CoV-2 nucleic acid and gene sequencing should be performed. ICH patients with SARS-CoV-2 infection are prone to develop neurological complications and have poor outcomes. Because there is no specific treatment for the virus, airborne precautions and isolation of identified and suspected infected patients is crucial.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

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REFERENCES

1. Jin YH, Cai L, Cheng ZS, Cheng H, Deng T, Fan YP, Fang C, Huang D, Huang LQ, Huang Q, Han Y, Hu B, Hu F, et al, for the Zhongnan Hospital of Wuhan University Novel Coronavirus Management and Research Team, Evidence-Based Medicine Chapter of China International Exchange and Promotive Association for Medical and Health Care (CPAM). A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). Mil Med Res. 2020; 7:4. https://doi.org/10.1186/s40779-020-0233-6 PMID:32029004

2. Habibzadeh P, Stoneman EK. The novel coronavirus: a bird’s eye view. Int J Occup Environ Med. 2020; 11:65–71. https://doi.org/10.15171/ijoem.2020.1921 PMID:32020915

3. Gorbalenya AE, Baker SC, Baric RS, de Groot RJ, Drosten C, Gulyaeva AA, Haagmans BL, Lauber C, Leontovich AM, Neuman BW, Penzar D, Perlman S, Poon LLM, et al. 2020.

4. https://www.thepaper.cn/newsDetail_forward_6319695.

5. Chen Y, Liu Q, Guo D. Emerging coronaviruses: genome structure, replication, and pathogenesis. J Med Virol. 2020; 92:418–23. https://doi.org/10.1002/jmv.25681 PMID:31967327

6. Bleibtreu A, Bertine M, Bertin C, Houhou-Fidouh N, Visseaux B. Focus on middle east respiratory syndrome coronavirus (MERS-CoV). Med Mal Infect. 2020; 50:243–51. https://doi.org/10.1016/j.medmal.2019.10.004 PMID:31727466

7. Hui DS, Zumla A. Severe acute respiratory syndrome: historical, epidemiologic, and clinical features. Infect Dis Clin North Am. 2019; 33:869–89. https://doi.org/10.1016/j.idc.2019.07.001 PMID:31668196

8. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al. China Novel Coronavirus Investigating and Research Team. A Novel Coronavirus from Patients with Pneumonia in

China, 2019. N Engl J Med. 2020; 382:727–733. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

9. Tian X, Li C, Huang A, Xia S, Lu S, Shi Z, Lu L, Jiang S, Yang Z, Wu Y, Ying T. Potent binding of 2019 novel coronavirus spike protein by a SARS coronavirus-specific human monoclonal antibody. Emerg Microbes Infect. 2020; 9:382–385. https://doi.org/10.1080/22221751.2020.1729069 PMID:32065055

10. Chan JF, Kok KH, Zhu Z, Chu H, To KK, Yuan S, Yuen KY. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerg Microbes Infect. 2020; 9:221–36. https://doi.org/10.1080/22221751.2020.1719902 PMID:31987001

11. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579:270–73. https://doi.org/10.1038/s41586-020-2012-7 PMID:32015507

12. Xu X, Chen P, Wang J, Feng J, Zhou H, Li X, Zhong W, Hao P. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. Sci China Life Sci. 2020; 63:457–60. https://doi.org/10.1007/s11427-020-1637-5 PMID:32009228

13. Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science. 2005; 310:676–79. https://doi.org/10.1126/science.1118391 PMID:16195424

14. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol. 2004; 203:631–37. https://doi.org/10.1002/path.1570 PMID:15141377

15. Kuhn JH, Li W, Choe H, Farzan M. Angiotensin-converting enzyme 2: a functional receptor for SARS coronavirus. Cell Mol Life Sci. 2004; 61:2738–43. https://doi.org/10.1007/s00018-004-4242-5 PMID:15549175

16. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, Ren R, Leung KS, Lau EH, Wong JY, Xing X, Xiang N, Wu Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med. 2020; 382:1199–207. https://doi.org/10.1056/NEJMoa2001316 PMID:31995857

17. Wu Z, McGoogan JM. Characteristics of and Important Lessons from the Coronavirus Disease 2019 (COVID-19) Outbreak in China. JAMA, 2020; 323:1239-42. https://doi.org/10.1001/jama.2020.2648 PMID: 32091533

18. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

19. Ding Y, Wang H, Shen H, Li Z, Geng J, Han H, Cai J, Li X, Kang W, Weng D, Lu Y, Wu D, He L, et al. The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. J Pathol. 2003; 200:282–9. https://doi.org/10.1002/path.1440 PMID:12845623

20. Ng DL, Al Hosani F, Keating MK, Gerber SI, Jones TL, Metcalfe MG, Tong S, Tao Y, Alami NN, Haynes LM, Mutei MA, Abdel-Wareth L, Uyeki TM, et al. Clinicopathologic, immunohistochemical, and ultrastructural findings of a fatal case of middle east respiratory syndrome coronavirus infection in the United Arab Emirates, april 2014. Am J Pathol. 2016; 186:652–58. https://doi.org/10.1016/j.ajpath.2015.10.024 PMID:26857507

21. Camacho E, LoPresti MA, Bruce S, Lin D, Abraham M, Appelboom G, Taylor B, McDowell M, DuBois B, Sathe M, Sander Connolly E. The role of age in intracerebral hemorrhages. J Clin Neurosci. 2015; 22:1867–70. https://doi.org/10.1016/j.jocn.2015.04.020 PMID:26375325

22. Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. Lancet Neurol. 2012; 11:720–31. https://doi.org/10.1016/S1474-4422(12)70104-7 PMID:22698888

23. Wu G, Bao X, Xi G, Keep RF, Thompson BG, Hua Y. Brain injury after intracerebral hemorrhage in spontaneously hypertensive rats. J Neurosurg. 2011; 114:1805–11. https://doi.org/10.3171/2011.1.JNS101530 PMID:21294617

24. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506.

https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

25. Hua Y, Keep RF, Hoff JT, Xi G. Brain injury after intracerebral hemorrhage: the role of thrombin and iron. Stroke. 2007; 38:759–62. https://doi.org/10.1161/01.STR.0000247868.97078.10 PMID:17261733

26. Naidech AM, Liebling SM, Rosenberg NF, Lindholm PF, Bernstein RA, Batjer HH, Alberts MJ, Kwaan HC. Early platelet transfusion improves platelet activity and may improve outcomes after intracerebral hemorrhage. Neurocrit Care. 2012; 16:82–87. https://doi.org/10.1007/s12028-011-9619-3 PMID:21837536

27. Wright JW, Harding JW. The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. Pflugers Arch. 2013; 465:133–51. https://doi.org/10.1007/s00424-012-1102-2 PMID:22535332

28. Baltatu OC, Campos LA, Bader M. Local renin-angiotensin system and the brain—a continuous quest for knowledge. Peptides. 2011; 32:1083–86. https://doi.org/10.1016/j.peptides.2011.02.008 PMID:21333703

29. Li F. Receptor recognition and cross-species infections of SARS coronavirus. Antiviral Res. 2013; 100:246–54. https://doi.org/10.1016/j.antiviral.2013.08.014 PMID:23994189

30. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, Zou W, Zhan J, Wang S, Xie Z, Zhuang H, Wu B, Zhong H, et al. Multiple organ infection and the pathogenesis of SARS. J Exp Med. 2005; 202:415–24. https://doi.org/10.1084/jem.20050828 PMID:16043521

31. Desforges M, Le Coupanec A, Dubeau P, Bourgouin A, Lajoie L, Dubé M, Talbot PJ. Human coronaviruses and other respiratory viruses: underestimated opportunistic pathogens of the central nervous system? Viruses. 2019; 12:14. https://doi.org/10.3390/v12010014 PMID:31861926

32. Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J Biol Chem. 2000; 275:33238–43. https://doi.org/10.1074/jbc.M002615200 PMID:10924499

33. Regenhardt RW, Mecca AP, Desland F, Ritucci-Chinni PF, Ludin JA, Greenstein D, Banuelos C, Bizon JL, Reinhard MK, Sumners C. Centrally administered angiotensin-(1-7) increases the survival of stroke-prone spontaneously hypertensive rats. Exp Physiol. 2014; 99:442–53. https://doi.org/10.1113/expphysiol.2013.075242 PMID:24142453

34. Rabelo LA, Alenina N, Bader M. ACE2-angiotensin-(1-7)-mas axis and oxidative stress in cardiovascular disease. Hypertens Res. 2011; 34:154–60. https://doi.org/10.1038/hr.2010.235 PMID:21124322

35. Xia H, Suda S, Bindom S, Feng Y, Gurley SB, Seth D, Navar LG, Lazartigues E. ACE2-mediated reduction of oxidative stress in the central nervous system is associated with improvement of autonomic function. PLoS One. 2011; 6:e22682. https://doi.org/10.1371/journal.pone.0022682 PMID:21818366

36. Mowry FE, Biancardi VC. Neuroinflammation in hypertension: the renin-angiotensin system versus pro-resolution pathways. Pharmacol Res. 2019; 144:279–91. https://doi.org/10.1016/j.phrs.2019.04.029 PMID:31039397

37. Kuba K, Imai Y, Ohto-Nakanishi T, Penninger JM. Trilogy of ACE2: a peptidase in the renin-angiotensin system, a SARS receptor, and a partner for amino acid transporters. Pharmacol Ther. 2010; 128:119–28. https://doi.org/10.1016/j.pharmthera.2010.06.003 PMID:20599443

38. Rocha NP, Simoes E Silva AC, Prestes TR, Feracin V, Machado CA, Ferreira RN, Teixeira AL, de Miranda AS. RAS in the central nervous system: potential role in neuropsychiatric disorders. Curr Med Chem. 2018; 25:3333–52. https://doi.org/10.2174/0929867325666180226102358 PMID:29484978

39. Kangussu LM, Marzano LA, Souza CF, Dantas CC, Miranda AS, Simões E Silva AC. The renin-angiotensin system and the cerebrovascular diseases: experimental and clinical evidence. Protein Pept Lett. 2020; 27:463–75. https://doi.org/10.2174/0929866527666191218091823 PMID:31849284

40. Shimada K, Furukawa H, Wada K, Wei Y, Tada Y, Kuwabara A, Shikata F, Kanematsu Y, Lawton MT, Kitazato KT, Nagahiro S, Hashimoto T. Angiotensin-(1-7) protects against the development of aneurysmal subarachnoid hemorrhage in mice. J Cereb Blood Flow Metab. 2015; 35:1163–68. https://doi.org/10.1038/jcbfm.2015.30 PMID:25757758

41. Becker LK, Etelvino GM, Walther T, Santos RA, Campagnole-Santos MJ. Immunofluorescence localization of the receptor mas in cardiovascular-

related areas of the rat brain. Am J Physiol Heart Circ Physiol. 2007; 293:H1416–24.
https://doi.org/10.1152/ajpheart.00141.2007
PMID:17496218

42. Ye R, Liu Z. ACE2 exhibits protective effects against LPS-induced acute lung injury in mice by inhibiting the LPS-TLR4 pathway. Exp Mol Pathol. 2020; 113:104350.
https://doi.org/10.1016/j.yexmp.2019.104350
PMID:31805278

43. Yagil Y, Yagil C. Hypothesis: ACE2 modulates blood pressure in the mammalian organism. Hypertension. 2003; 41:871–73.
https://doi.org/10.1161/01.HYP.0000063886.71596.C8
PMID:12654716

44. Jiang T, Gao L, Lu J, Zhang YD. ACE2-ang-(1-7)-mas axis in brain: a potential target for prevention and treatment of ischemic stroke. Curr Neuropharmacol. 2013; 11:209–17.
https://doi.org/10.2174/1570159X11311020007
PMID:23997755

45. Peña Silva RA, Chu Y, Miller JD, Mitchell IJ, Penninger JM, Faraci FM, Heistad DD. Impact of ACE2 deficiency and oxidative stress on cerebrovascular function with aging. Stroke. 2012; 43:3358–63.
https://doi.org/10.1161/STROKEAHA.112.667063
PMID:23160880

46. Saavedra JM, Sánchez-Lemus E, Benicky J. Blockade of brain angiotensin II AT1 receptors ameliorates stress, anxiety, brain inflammation and ischemia: therapeutic implications. Psychoneuroendocrinology. 2011; 36:1–18.
https://doi.org/10.1016/j.psyneuen.2010.10.001
PMID:21035950

47. Kikkert M. Innate immune evasion by human respiratory RNA viruses. J Innate Immun. 2020; 12:4–20.
https://doi.org/10.1159/000503030
PMID:31610541

48. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, Pan P, Wang W, Hu D, Liu X, Zhang Q, Wu J. Coronavirus infections and immune responses. J Med Virol. 2020; 92:424–432.
https://doi.org/10.1002/jmv.25685
PMID:31981224

49. Perlman S, Dandekar AA. Immunopathogenesis of coronavirus infections: implications for SARS. Nat Rev Immunol. 2005; 5:917–27.
https://doi.org/10.1038/nri1732 PMID:16322745

50. Trottein F, Paget C. Natural killer T cells and mucosal-associated invariant T cells in lung infections. Front Immunol. 2018; 9:1750.
https://doi.org/10.3389/fimmu.2018.01750
PMID:30116242

51. Pascal KE, Coleman CM, Mujica AO, Kamat V, Badithe A, Fairhurst J, Hunt C, Strein J, Berrebi A, Sisk JM, Matthews KL, Babb R, Chen G, et al. Pre- and postexposure efficacy of fully human antibodies against spike protein in a novel humanized mouse model of MERS-CoV infection. Proc Natl Acad Sci USA. 2015; 112:8738–43.
https://doi.org/10.1073/pnas.1510830112
PMID:26124093

52. Romero-Brey I, Bartenschlager R. Membranous replication factories induced by plus-strand RNA viruses. Viruses. 2014; 6:2826–57.
https://doi.org/10.3390/v6072826
PMID:25054883

53. Gralinski LE, Baric RS. Molecular pathology of emerging coronavirus infections. J Pathol. 2015; 235:185–95.
https://doi.org/10.1002/path.4454
PMID:25270030

54. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med. 2020; 8:420–22.
https://doi.org/10.1016/S2213-2600(20)30076-X
PMID:32085846

55. Zhao J, Zhao J, Mangalam AK, Channappanavar R, Fett C, Meyerholz DK, Agnihothram S, Baric RS, David CS, Perlman S. Airway Memory CD4(+) T Cells Mediate Protective Immunity against Emerging Respiratory Coronaviruses. Version 2. Immunity. 2016; 44:1379–91.
https://doi.org/10.1016/j.immuni.2016.05.006
PMID:27287409

56. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Semin Immunopathol. 2017; 39:529–39.
https://doi.org/10.1007/s00281-017-0629-x
PMID:28466096

57. Law HK, Cheung CY, Ng HY, Sia SF, Chan YO, Luk W, Nicholls JM, Peiris JS, Lau YL. Chemokine up-regulation in SARS-coronavirus-infected, monocyte-derived human dendritic cells. Blood. 2005; 106:2366–74.
https://doi.org/10.1182/blood-2004-10-4166
PMID:15860669

58. Liu J, Zheng X, Tong Q, Li W, Wang B, Sutter K, Trilling M, Lu M, Dittmer U, Yang D. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. J Med Virol. 2020; 92:491–94.
https://doi.org/10.1002/jmv.25709

PMID:32056249

59. Wong CK, Lam CW, Wu AK, Ip WK, Lee NL, Chan IH, Lit LC, Hui DS, Chan MH, Chung SS, Sung JJ. Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. Clin Exp Immunol. 2004; 136:95–103. https://doi.org/10.1111/j.1365-2249.2004.02415.x PMID:15030519

60. Shin HS, Kim Y, Kim G, Lee JY, Jeong I, Joh JS, Kim H, Chang E, Sim SY, Park JS, Lim DG. Immune responses to middle east respiratory syndrome coronavirus during the acute and convalescent phases of human infection. Clin Infect Dis. 2019; 68:984–92. https://doi.org/10.1093/cid/ciy595 PMID:30060038

61. Mahallawi WH, Khabour OF, Zhang Q, Makhdoum HM, Suliman BA. MERS-CoV infection in humans is associated with a pro-inflammatory Th1 and Th17 cytokine profile. Cytokine. 2018; 104:8–13. https://doi.org/10.1016/j.cyto.2018.01.025 PMID:29414327

62. Zhang Y, Li J, Zhan Y, Wu L, Yu X, Zhang W, Ye L, Xu S, Sun R, Wang Y, Lou J. Analysis of serum cytokines in patients with severe acute respiratory syndrome. Infect Immun. 2004; 72:4410–5. https://doi.org/10.1128/IAI.72.8.4410-4415.2004 PMID:15271897

63. Prompetchara E, Ketloy C, Palaga T. Immune responses in COVID-19 and potential vaccines: lessons learned from SARS and MERS epidemic. Asian Pac J Allergy Immunol. 2020; 38:1–9. https://doi.org/10.12932/AP-200220-0772 PMID:32105090

64. Tschoe C, Bushnell CD, Duncan PW, Alexander-Miller MA, Wolfe SQ. Neuroinflammation after intracerebral hemorrhage and potential therapeutic targets. J Stroke. 2020; 22:29–46. https://doi.org/10.5853/jos.2019.02236 PMID:32027790

65. Sansing LH, Harris TH, Kasner SE, Hunter CA, Kariko K. Neutrophil depletion diminishes monocyte infiltration and improves functional outcome after experimental intracerebral hemorrhage. Acta Neurochir Suppl. 2011; 111:173–78. https://doi.org/10.1007/978-3-7091-0693-8_29 PMID:21725751

66. Tejima E, Zhao BQ, Tsuji K, Rosell A, van Leyen K, Gonzalez RG, Montaner J, Wang X, Lo EH. Astrocytic induction of matrix metalloproteinase-9 and edema in brain hemorrhage. J Cereb Blood Flow Metab. 2007; 27:460–68. https://doi.org/10.1038/sj.jcbfm.9600354 PMID:16788715

67. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol. 2010; 92:463–77. https://doi.org/10.1016/j.pneurobio.2010.08.001 PMID:20713126

68. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. Stroke. 2011; 42:1781–86. https://doi.org/10.1161/STROKEAHA.110.596718 PMID:21527759

69. Wilkinson DA, Pandey AS, Thompson BG, Keep RF, Hua Y, Xi G. Injury mechanisms in acute intracerebral hemorrhage. Neuropharmacology. 2018; 134:240–48. https://doi.org/10.1016/j.neuropharm.2017.09.033 PMID:28947377

70. Hwang BY, Appelboom G, Ayer A, Kellner CP, Kotchetkov IS, Gigante PR, Haque R, Kellner M, Connolly ES. Advances in neuroprotective strategies: potential therapies for intracerebral hemorrhage. Cerebrovasc Dis. 2011; 31:211–22. https://doi.org/10.1159/000321870 PMID:21178344

71. McCray PB Jr, Pewe L, Wohlford-Lenane C, Hickey M, Manzel L, Shi L, Netland J, Jia HP, Halabi C, Sigmund CD, Meyerholz DK, Kirby P, Look DC, Perlman S. Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. J Virol. 2007; 81:813–21. https://doi.org/10.1128/JVI.02012-06 PMID:17079315

72. Arabi YM, Harthi A, Hussein J, Bouchama A, Johani S, Hajeer AH, Saeed BT, Wahbi A, Saedy A, AlDabbagh T, Okaili R, Sadat M, Balkhy H. Severe neurologic syndrome associated with middle east respiratory syndrome corona virus (MERS-CoV). Infection. 2015; 43:495–501. https://doi.org/10.1007/s15010-015-0720-y PMID:25600929

73. Li K, Wohlford-Lenane C, Perlman S, Zhao J, Jewell AK, Reznikov LR, Gibson-Corley KN, Meyerholz DK, McCray PB Jr. Middle east respiratory syndrome coronavirus causes multiple organ damage and lethal disease in mice transgenic for human dipeptidyl peptidase 4. J Infect Dis. 2016; 213:712–22. https://doi.org/10.1093/infdis/jiv499 PMID:26486634

74. Bleau C, Filliol A, Samson M, Lamontagne L. Brain invasion by mouse hepatitis virus depends on impairment of tight junctions and beta interferon production in brain microvascular endothelial cells. J Virol. 2015; 89:9896–908.

https://doi.org/10.1128/JVI.01501-15
PMID:26202229

75. Huang KJ, Su IJ, Theron M, Wu YC, Lai SK, Liu CC, Lei HY. An interferon-gamma-related cytokine storm in SARS patients. J Med Virol. 2005; 75:185–94. https://doi.org/10.1002/jmv.20255
PMID:15602737

Research Paper

High neutrophil-to-lymphocyte ratio associated with progression to critical illness in older patients with COVID-19: a multicenter retrospective study

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ABSTRACT

This retrospective cohort study aimed to investigate the correlation of the neutrophil-to-lymphocyte ratio (NLR) with critical illness in older patients with COVID-19, and evaluate the prognostic power of the NLR at admission. We enrolled 232 patients with COVID-19, aged ≥60 y, in Zhejiang province from January 17 to March 3, 2020. Primary outcomes were evaluated until April 13. Cox regression was performed for prognostic factors. Twenty-nine (12.5%) patients progressed to critical illness. Age, shortness of breath, comorbidities including hypertension, heart disease, and chronic obstructive pulmonary disease, higher NLR, lower albumin levels, and multiple mottling and ground-glass opacity were associated with progression. In the multivariate analysis, older age (hazard ratio [HR] 1.121, confidence interval [CI] 1.070-1.174, P<0.001), heart disease (HR 2.587, CI 1.156-5.787, P=0.021), higher NLR (HR 1.136, CI 1.094-1.180, P < 0.001), and multiple mottling and ground-glass opacity (HR 4.518, CI 1.906-10.712, P<0.001) remained critical illness predictors. The NLR was independently associated with progression to critical illness; the relationship was significant and graded (HR: 1.16 per unit; 95% CI: 1.10-1.22; P for trend < 0.001). Therefore, NLR can be adopted as a prognostic tool to assist healthcare providers predict the clinical outcomes of older patients suffering from COVID-19.

INTRODUCTION

In December 2019, a novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified in Wuhan, China [1–3]. Infection with the virus leads to coronavirus disease (COVID-19), which is characterized by rapid human-to-human transmission and varied degrees of fatality, due to acute respiratory distress syndrome, multi-organ failure, and other serious complications [4, 5]. The global spread of this pandemic has been rapid since March 2020. As of mid-April 2020, more than 2 million individuals had been diagnosed with the disease, leading to over 150,000 deaths.

In our previous study, we found that older patients with COVID-19 had significantly greater disease severities, as well as higher rates of critical-type disease and intensive care unit (ICU) admission than their younger counterparts outside Wuhan [6]. Wang et al. [7] found that patients treated in the ICU were older than those without ICU treatment in Wuhan. In the United States, Garg et al. [8] demonstrated that older adults had elevated rates of COVID-19-associated hospitalization, and the majority of people hospitalized with COVID-19 had underlying medical conditions. In Italy, a majority of critically ill patients with laboratory-confirmed COVID-19 who were admitted to ICUs were older men, and a large proportion of them required mechanical ventilation and high levels of positive end-expiratory pressure; the associated ICU-related mortality was 26% [9].

Many studies have shown that older age is an independent risk factor for fatal outcomes in patients with COVID-19 [10–12]. Wang et al. investigated the characteristics of elderly patients with COVID-19 and the associated prognostic factors, and found that the presence of acute respiratory distress syndrome was a strong predictor of death. In addition, high lymphocyte levels were predictive of better outcomes [13]. Lymphopenia is a risk factor for severe illness and death among patients with COVID-19 [14].

The neutrophil-to-lymphocyte ratio (NLR) can be easily determined from the full blood count, and has been reported to be closely related to patients' overall inflammatory status.

Increasing NLR values are risk factors of mortality in not only infectious disease settings but also cancer [15, 16]. A study showed that the NLR is an independent risk factor of mortality in hospitalized patients with COVID-19 [17]. The identification of a good indicator of disease progression can aid clinicians in improving the effect of therapy and reducing the mortality related to COVID-19 without excessive medical resource use. Whether the NLR can predict progression to critical illness in older patients with COVID-19 requires further elucidated.

In this study, we investigated the correlation of the NLR with critical illness in older patients with COVID-19, to evaluate the prognostic power of the NLR at admission in the prediction of progression to critical illness.

RESULTS

Demographic and epidemiologic characteristics

In this study, 232 older (≥60 years) patients with confirmed COVID-19 were enrolled from January 17, 2020 to March 3, 2020 in Zhejiang province. Patients' clinical outcomes were followed-up until April 13, 2020. As shown in Table 1, the median ages in the mild, severe, and critical disease groups were 66 years (interquartile range [IQR]: 63-70), 66 years (IQR: 62-71) and 72 years (IQR: 68-81). The critical group showed a significantly higher age than the mild and severe groups (P<0.001). The proportions of hypertension and heart disease in the critical group were 72.41% and 55.17%, respectively, which were significantly higher than those noted in the mild and severe groups (P<0.001). One case (0.71%) with mild disease, two (3.14%) with severe disease, and six (20.69%) with critical disease had chronic obstructive pulmonary disease (COPD) (P<0.001). There were no significant differences in the other coexisting medical conditions across the three groups, including the rates of diabetes, asthma, cancer, chronic liver disease, chronic renal disease, and immunosuppression.

Clinical features and laboratory abnormalities

On admission, the majority of cases showed decreased or normal leucocyte levels in all subtypes, as shown in Table 2. The median neutrophil levels in the mild, severe, and critical groups were 3.22×10^9 /L [IQR: (2.59-4.20) $\times10^9$], 3.50×10^9/L [IQR: (2.70-4.80) $\times10^9$], and 6.65×10^9/L [IQR: (3.51-9.70) $\times10^9$], respectively; the critical group showed significantly higher values than the mild and severe groups (P<0.001). The median lymphocyte levels in the mild, severe, and critical groups were 1.26×10^9 /L [IQR: (0.90-1.60) $\times10^9$], 0.98×10^9/L [IQR: (0.70-1.26) $\times10^9$], and 0.54×10^9/L [IQR: (0.45-0.80) $\times10^9$], respectively. The critical group showed significantly lower values than the mild and severe groups (P<0.001). The platelet levels were lower in the critically group than the mild and severe groups, but were still within the normal range. The levels of lactate dehydrogenase, creatinine, C-reactive protein, and procalcitonin increased with increasing illness severity (P<0.05). There were no significant differences in the blood test results across the three groups, including the values of albumin, alanine aminotransferase, aspartate aminotransferase, total bilirubin, potassium, sodium, and blood urea nitrogen. Multiple mottling and ground-glass opacity were typical imaging manifestations noted in patients with COVID-19, and their prevalence rates in the mild, severe, and critical groups were 24.29%, 42.86%, and 68.97%, respectively (P<0.001).

Treatment and outcomes

All patients were isolated in designated hospitals and received supportive care as well as the currently recommended medications. As shown in Table 3, 135 cases (84.77%), 60 cases (95.24%), and 29 cases (100%) received antiviral treatment, including interferon-α sprays, arbidol hydrochloride capsules, and

Table 1. Demographic, epidemiologic, and clinical characteristics of the different subtypes in older patients with COVID-19.

Characteristic	Mild type (n=140)	Severe type (n=63)	Critical type (n=29)	*P* value
Age (years)	66(63-70)	66(62-71)	72(68-81)	<0.001
Distribution				
60-70 y	102(72.86)	45(71.435)	7(24.14)	<0.001
70-80 y	30(21.43)	14(22.22)	13(44.83)	0.025
≥80 y	8(5.71)	4(6.35)	9(31.03)	<0.001
Sex (male)	62(44.29)	28(44.44)	19(65.52)	0.102
Body mass index (kg/m^2)	23.52(21.23-25.39)	24.34(22.25-25.16)	24.51(22.89-26.62)	0.227
Current smoker	17(12.14)	4(6.35)	4(13.79)	0.418
Exposure history in Wuhan	25(17.86)	18(28.57)	5(17.24)	0.194
Contact with patients	82(57.14)	25(39.68)	12(41.37)	0.023
Family cluster	50(35.71)	20(31.75)	10(34.48)	0.859
Time from illness onset to first hospital admission (days)	3(1-6)	5(2-7)	3(1-5)	0.048
Coexisting disorder				
Any	76(54.29)	25(38.68)	13(44.83)	0.132
Hypertension	57(40.71)	22(34.92)	21(72.41)	0.004
Heart disease	8(5.71)	7(11.11)	16(55.17)	<0.001
Diabetes	29(20.71)	9(14.29)	4(13.79)	0.431
asthma	1(0.71)	1(1.59)	2(6.90)	0.076
Chronic obstructive pulmonary disease	1(0.71)	2(3.14)	6(20.69)	<0.001
Cancer	2(14.29)	1(1.59)	1(3.45)	0.766
Chronic liver disease	4(2.86)	4(6.35)	2(6.90)	0.397
Chronic renal disease	3(2.14)	1(1.59)	2(6.90)	0.313
Immunosuppression	0(0)	2(3.17)	0(0)	0.064
Symptoms on admission				
Fever	110(78.57)	55(87.30)	25(86.21)	0.105
Cough	94(67.14)	38(60.2)	22(75.87)	0.461
Sputum production	46(32.86)	26(41.27)	15(51.72)	0.148
Hemoptysis	2(1.43)	1(1.59)	1(3.45)	0.766
Sore throat	13(9.29)	8(12.70)	2(6.90)	0.598
Nasal obstruction	2(1.43)	0(0%)	1(3.45)	0.404
Myalgia	12(8.57)	8(12.70)	4(13.79)	0.552
Fatigue	19(13.57)	11(17.46)	8(27.59)	0.202
Gastrointestinal symptoms	12(8.57)	6(9.52)	7(24.14)	0.06
Headache	5(3.57)	6(9.52)	0(0%)	0.073
Shortness of breath	1(0.71)	7(11.11)	12(41.38)	<0.001

Data are presented as medians (interquartile ranges), n (%) and n/N (%).

lopinavir and ritonavir tablets in the mild, severe, and critical groups, respectively (*P*=0.504). The durations from illness onset to antiviral therapy initiation were 4 days (IQR: 2.0-7.0), 5 days (IQR: 1.5-8.5), and 4 days (IQR: 2.0-8.0) in the mild, severe, and critical groups, respectively (*P*=0.390). With increases in the illness severity, the proportion of the use of glucocorticoids and intravenous immunoglobins rose (*P*<0.001). Ten

Table 2. Laboratory and radiograph findings of the different subtypes in older patients with COVID-19.

Characteristic	Mild type (n=140)	Severe type (n=63)	Critical type (n=29)	*P* value
Blood routine				
Leucocyte count ($\times 10^9$/L)	5.20(4.38-6.48)	5.0(4.1-6.88)	8.08(4.4-10.8)	0.02
Neutrophil count ($\times 10^9$/L)	3.22(2.59-4.20)	3.50(2.70-4.80)	6.65(3.51-9.70)	<0.001
Lymphocyte count ($\times 10^9$/L)	1.26(0.90-1.60)	0.98(0.70-1.26)	0.54(0.45-0.80)	<0.001
Neutrophil count/lymphocyte count	2.45(1.82-3.65)	4.08(2.39-6.20)	9.67(6.86-21.10)	<0.001
Hemoglobin (g/L)	125.0(113.0-138.0)	122.0(113.5-133.5)	121.0(110.5-137.5)	0.535
Platelet count ($\times 10^9$/L)	204(170-279)	175(139-236)	156(123-191)	<0.001
Coagulation function				
International normalized ratio	1.02(0.96-1.06)	1.01(0.96-1.10)	1.0(0.97-1.06)	0.895
Blood biochemistry				
Albumin (g/L)	38.40(35.43-41.25)	36.30(33.30-39.50)	34.60(30.65-38.45)	0.001
Alanine aminotransferase (U/L)	25(16-36)	24(16-31)	21(14-31)	0.664
Aspartate aminotransferase (U/L)	25(20-33)	25(19-34)	29(18-38)	0.891
Total bilirubin (umol//L)	9.70(7.0-12.55)	10.10(7.90-13.15)	9.10(5.70-14.30)	0.671
Potassium (mmol/L)	3.99(3.70-4.37)	3.89(3.45-4.25)	3.81(3.50-4.14)	0.072
Sodium (mmol/L)	138.0(135.72-140.15)	137.50(134.95-140.0)	136.0(130.60-139.0)	0.027
Blood urea nitrogen (mmol/L)	4.51(3.83-5.47)	4.59(3.60-7.10)	6.16(4.48-8.72)	0.032
Creatinine (umol/L)	64.0(54.0-76.5)	68.0(57.0-84.0)	76.0(63.0-96.5)	0.003
Creatinine kinase (U/L)	56.50(41.25-88.75)	62.0(26.25-113.75)	80.0(52.0-173.50)	0.038
Lactate dehydrogenase (U/L)	218.0(175.0-256.50)	233.0(190.0-313.0)	273.0(243.0-354.0)	<0.001
Infection-related biomarkers				
C-reactive protein (mg/L)	16.02(4.41-39.26)	19.10(5.89-44.70)	41.86(6.33-70.10)	0.039
Procalcitonin (ng/mL)	0.09(0.04-0.14)	0.05(0.04-0.08)	0.19(0.04-0.25)	0.046
Chest radiography/Computed tomography findings				
Multiple mottling and ground-glass opacity	34(24.29)	27(42.86)	20(68.97)	<0.001

Data are presented as medians (interquartile ranges), n (%) and n/N (%).

patients received extracorporeal membrane oxygenation (ECMO) therapy, and six underwent continuous renal-replacement therapy (CRRT) in the critical group; none of the patients received ECMO therapy and only one underwent CRRT in the severe group. Three patients had shock in the critical group, while there were no cases with shock in the mild and severe groups (*P*<0.001). The viral RNA shedding durations were 16 days (IQR: 12-22), 17 days (IQR: 14-21), and 25 days (IQR: 17-30) in the mild, severe, and critical groups, respectively (P<0.001).

By April 13, one patient had died, two had received lung transplantation, and eight remained hospitalized in the critical group. By May 27, among the eight patients who were still hospitalized, two withdrew from the ECMO treatment and were transferred to the general ward, while the other six patients were still receiving the ECMO therapy. In the other two groups, all patients had survived and were discharged. The number of days of hospitalization were 18 days [IQR: 14-23], 22 days [IQR: 19-26], and 32 days [IQR: 21-68] in the mild, severe, and critical groups, respectively (P<0.001).

Risk factors associated with progression to critical illness

Univariate Cox regression was used to analyze the risk factors for critical illness in the older patients with COVID-19, as shown in Table 4. Older age was shown to increase the likelihood of critical illness even in older patients (≥60 years) (hazard ratio [HR] 1.107, confidence interval [CI] 1.065-1.151, *P*<0.001). Shortness of breath as a symptom (HR 11.328,

Table 3. Treatments and clinical outcomes of the different subtypes in older patients with COVID-19.

Characteristic	Mild type (n=140)	Severe type (n=63)	Critical type (n=29)	*P* value
Shock	0(0)	0(0)	3(10.34)	<0.001
Time from illness onset to antiviral treatment initiation (days)	4.0(2.0-7.0)	5.0(1.5-8.5)	4.0(2.0-8.0)	0.390
Antiviral treatment	135(96.43)	60(95.24)	29(100)	0.504
Viral RNA shedding time	16(12-22)	17(14-21)	25(17-30)	<0.001
Glucocorticoids	22(15.71)	29(46.03)	26(89.66)	<0.001
Use of intravenous immunoglobulin	17()	21()	23(79.31)	<0.001
Use of extracorporeal membrane oxygenation	0(0)	0(0)	10(34.48)	<0.001
Use of continuous renal-replacement therapy	0(0)	1(1.59)	6(20.69)	<0.001
Clinical outcomes at data cutoff				
Discharge from hospital	140(100)	63(100)	20(68.97)	0.098
Hospitalization	0(0)	0(0)	8(27.59)	0.098
Number of days in hospital	18(14-23)	22(19-26)	32(21-68)	<0.001
Lung transplantation	0(0)	0(0)	2(6.90)	0.001
Death	0(0)	0(0)	1(3.45)	0.030

Data are presented as medians (interquartile ranges), n (%) and n/N (%).

Table 4. Risk factors for critical illness.

Variables	Mild/Severe type (n=203)	Critical type (n=29)	Univariate analysis		Multivariate analysis	
			HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)	66(63-70)	72(68-81)	1.107(1.065-1.151)	<0.001	1.121(1.070-1.174)	<0.001
Time from illness onset to first hospital admission (days)	3(1-7)	3(1-5)	0.937(0.836-1.049)	0.258		
Hypertension	79(38.92)	21(72.41)	3.563(1.578-8.047)	0.002		
Heart disease	15(7.39)	16(55.17)	9.638(4.626-20.081)	<0.001	2.587(1.156-5.787)	0.021
COPD	3(1.48)	6(20.69)	7.108(2.891-17.481)	<0.001		
Shortness of breath	8(3.94)	12(41.38)	11.328(5.370-23.894)	<0.001		
NLR	2.68(1.96-4.42)	9.67(6.86-21.10)	1.157(1.117-1.199)	<0.001	1.136(1.094-1.180)	<0.001
Albumin (g/L)	38.0(35.20-41.0)	34.60(30.65-38.45)	0.875(0.807-0.950)	0.001		
C-reactive protein (mg/L)	16.95(4.75-40.62)	41.86(6.33-70.10)	1.012(1.005-1.020)	0.002		
Multiple mottling and ground-glass opacity	61(30.05)	20(68.97)	4.573(2.082-10.045)	<0.001	4.518 (1.906-10.712)	0.001

HR, hazard ratio; CI, confidence interval; COPD, chronic obstructive pulmonary disease; NLR, neutrophil-to-lymphocyte ratio.

CI 5.370-23.894, P<0.001), and comorbidities including hypertension (HR 3.563, CI 1.578-8.047, *P*=0.002), heart disease (HR 9.638, CI 4.626-20.081, *P*<0.001), and COPD (HR 7.108, CI 2.891-17.481, *P*<0.001) were predictive of critical illness. The increasing odds of critical illness development in patients with COVID-19 were associated with higher NLR values (HR 1.157, CI 1.117-1.199, *P*<0.001), lower albumin levels (HR 0.875, CI 0.807-0.950, *P*<0.001), higher C-reactive protein levels (HR 1.012, CI 1.005-1.020, *P*=0.002), and multiple mottling and ground-glass opacity (HR 4.573, CI 2.082-10.045, P<0.001). In the multivariate analysis, only older age (HR 1.121, CI 1.070-1.174, *P*<0.001), heart disease (HR 2.587, CI 1.156-5.787, *P*=0.021), higher NLRs (HR 1.136, CI 1.094-1.180, *P* < 0.001), and multiple mottling and ground-glass opacity (HR 4.518, CI 1.906-10.712, P<0.001) remained predictors of critical illness when the other factors in the model were kept constant.

Association of the NLR with progression to critical illness

Figure 1A shows the association between the NLR and progression to critical illness, as identified using a Cox proportional hazards model adjusted for the baseline covariates. For the sensitivity analysis, we converted the NLR from a continuous variable to a categorical

variable (the quartile of NLR), and the P for trend of the NLR with categorical variables in the fully adjusted model (model II) was consistent with that obtained when the NLR was a continuous variable. The relationship between the NLR and progression was significant and graded (HR: 1.16 per unit; 95% CI: 1.10-1.22; P<0.001). When adjusted for sex and age, the ratio of the highest quartile of the NLR compared to the lowest quartile was 33.017 (95% CI 4.436-245.732, P <0.001), and in the fully adjusted model, the odds of the NLR as a clinical risk factor was 21.755 (95% CI 2.854-165.860, P<0.001) (Table 5). Figure 1B shows the Kaplan-Meier analyses graphs for progression to critical illness based on the quartiles of the NLR.

DISCUSSION

In the present study, we described the clinical characteristics and outcomes of older patients who had COVID-19 with the highest risk of critical illness after SARS-CoV-2 infection. Of the 232 older patients with COVID-19, 29 (12.5%) had critical disease; one patient died and two received lung transplantation in the critical group. Eight patients remained in the hospital, and received ECMO therapy for more than two weeks. The median duration of hospitalization was 32 days in the critical group, which was significantly longer than that in the mild and severe groups.

Disease typing and prognostic indicators are of great significance in the guidance of classified treatment and prevention of medical runs, and saving patients with a critical status. In our study, some independent risk factors for progression to critical illness were found using multivariate Cox regression analysis, such as older age, multiple mottling and ground-glass opacity, heart disease, and a high NLR.

Previously, older age was reported as an important independent predictor of fatal outcomes in patients with COVID-19 [18–21]. Older age was shown to increase the likelihood of critical illness even in older patients (HR 1.107, CI 1.065-1.151, P<0.001). Our results are consistent with those of previous reports [13]. Elderly patients experience a marked cell-mediated immune function decline and a reduced degree of humoral immune function. The cytokine and chemokine signaling networks are altered in elderly patients, and tend to favor a type 2 cytokine response over type 1 cytokine responses, potentially leading to poor outcomes [22].

Advanced imaging in patients with COVID-19 is capable of demonstrating disease progression. Generally, imaging manifestations are in line with the severity of COVID-19 [23]. Zhong et al. found that the computed tomography (CT) images in patients with different clinical types of COVID-19 had characteristic manifestations, and that the presence of solid shadows may be predictive of severe and critical illness [24]. Our study found that the presence of multiple mottling and ground-glass opacity on CT was an independent predictor of progression to critical illness (HR 4.518, CI

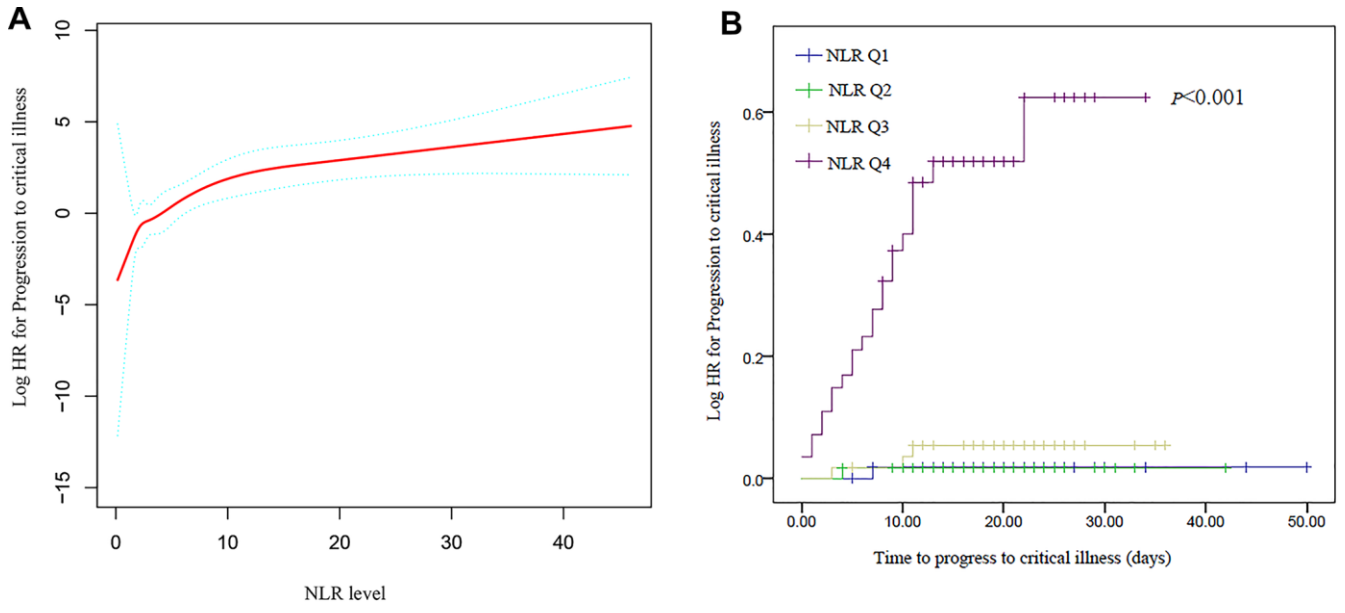

Figure 1. Association between the neutrophil-to-lymphocyte ratio (NLR) and progression to critical illness. (**A**) Adjusted hazard ratio (HR) for progression to critical illness according to the NLR. (**B**) Cumulative probability of progression to critical illness with increasing NLR values.

Table 5. Relationships between the neutrophil-to-lymphocyte ratio and critical disease development using different models.

Neutrophil-to-lymphocyte ratio (quartile)	Total, n	Event (%)	HR (95% CI)		
			Crude Model	Model I	Model II
Q1	58	1(1.72)	Reference	Reference	Reference
Q2	60	1(1.61)	0.980(0.061-15.662)	1.186(0.074-18.984)	1.324(0.081-21.591)
Q3	57	3(5.26)	2.914(0.303-28.014)	2.966(0.308-28.533)	3.867(0.399-37.461)
Q4	57	24(42.11)	29.769(4.024-220.233)	33.017(4.436-245.732)	21.755(2.854-165.860)
P for trend	—	—	<0.001	<0.001	<0.001
Increase per unit	—	—	1.16(1.12-1.20)	1.15(1.11-1.19)	1.16(1.10-1.22)

Note: Model I adjusted for age, sex.
Model II adjusted for age, sex, hypertension, heart disease, COPD, shortness of breath, albumin, C-reactive protein and multiple mottling and ground-glass opacity.
HR, hazard ratio; CI, confidence interval; COPD, chronic obstructive pulmonary disease.

1.906-10.712, *P*=0.001). We also found that older patients with COVID-19 who had heart disease were likelier to progress to critical illness. Several studies have shown that coexisting heart disease was an independent risk factor associated with fatal outcomes in patients with COVID-19 [12, 25]. Cardiac complications, including new or worsening heart failure, new or worsening arrhythmia, and myocardial infarction are commonly observed in patients with severe pneumonia. Cardiac arrest occurs in about 3% of inpatients with severe pneumonia [26].

Chen et al. showed that, compared to cases with moderate disease severity, those with a severe disease status more frequently had lymphopenia [27]. Mo et al. found that patients with refractory disease had higher neutrophil levels than general COVID-19 patients [28]. The prognostic role of the NLR has been documented in multiple settings, including malignancies, infectious diseases, liver cirrhosis, and cerebrovascular disease [29–32]. In this study, we investigated the correlation of the NLR with critical illness in older patients with COVID-19 to evaluate the prognostic power of the NLR at admission in the prediction of progression to critical illness. In the sensitivity analysis, we converted the NLR from a continuous variable to a categorical variable, and found that the higher the NLR the greater the likelihood of progression to critical illness. Liu et al. also found that the NLR is an independent risk factor of in-hospital mortality in COVID-19 patients, especially male patients [17]. Our previous study suggested that a change in the NLR on admission among older patients with COVID-19 might be a biomarker specific to the prediction of progression to critical illness. A future study, conducted to elucidate this specificity, will further our understanding of the prognostic value of the NLR.

Our study has several limitations. First, its retrospective nature may decrease the accuracy of the findings; there is a need for a validation cohort to assess the predictive accuracy and confirm our findings. Second, owing to the retrospective design, data on some relevant factors such as interleukin-6 and D-dimer were incomplete and could not be included in the risk factor analysis. Third, data on the outcomes of older patients with COVID-19 in the critical group require further investigation, as, at the time of this study, there were still eight patients who were undergoing treatment at the hospital.

MATERIALS AND METHODS

Patients

This retrospective study, focusing on the epidemiological and clinical characteristics of older (age≥60 years) patients with confirmed COVID-19, was conducted from January 17 to March 3, 2020. All the enrolled cases showed real-time reverse transcriptase polymerase chain reaction (RT-PCR) positivity for SARS-CoV-2, and were retested several times during their hospitalization. Data were collected uniformly by the Health Commission of Zhejiang Province, wherein all patients were assigned to specific hospitals for unified treatment according to Zhejiang Province's emergency rule. The diagnosis of COVID-19 infection was based on the interim guidance of the World Health Organization (WHO) [33], and all data were shared with the WHO, with the primary analytic results reported to the authority of Zhejiang Province. Since the collection and analysis of all cases were determined by the Health Commission of Zhejiang Province under national authorization and considered as part of the continuing public health outbreak investigation, our retrospective study was exempt from institutional review board approval.

The subtype definition of COVID-19 patients was based on the diagnosis and treatment scheme for COVID-19 in China, based on a minor modification of WHO standards [34]. The degree of COVID-19 was categorized as mild, severe, or critical: the mild type included non-pneumonia and mild pneumonia cases, and the severe type was characterized by dyspnea, respiratory frequency ≥30 min, blood oxygen saturation ≤93%, PaO2/FiO2 ratio <300, and/or rate of lung infiltration >50% within 24–48 h. Critical cases were those that exhibited respiratory failure, septic shock, and/or multiple organ dysfunction/failure.

Procedures

We obtained epidemiological, demographic, laboratory, clinical, management, and outcome data from patients' medical records. Data were retrieved and reviewed by two independent observers. Clinical outcomes were followed-up until April 13, 2020. Missing or unclear data were confirmed by direct communication with healthcare providers. Throat swab specimens obtained from the upper respiratory tract and sputum of all patients were collected at admission. Laboratory confirmation of COVID-19 was performed at the First Affiliated Hospital at Zhejiang University, under the authorization of the Centers for Disease Control and Prevention at the Zhejiang Province/city level, by previously reported RT-PCR methods. All patients underwent chest CT at admission. Patients with other common respiratory viruses, including respiratory syncytial virus, parainfluenza virus, influenza A and B virus, and adenovirus were excluded from this study.

Data collection

In this study, we collected data on epidemiology, anthropometrics, demographics, as well as symptoms and signs at the time of admission to the hospital. We analyzed the blood collected within 48 hours of admission. Additional data collected included those on the results of laboratory tests and chest CT, comorbidities, co-infection with other respiratory pathogens, treatment (including drugs, intensive care and mechanical ventilation), and other clinical outcomes.

Statistical analysis

Continuous variables are expressed as medians (range), and were compared using t tests or Mann-Whitney U tests, and categorical variables were compared using chi-squared tests or Fisher's exact tests. Follow-up was initiated on the day of admission, and ended at the patient's death or until the last follow-up. The Kaplan-Meier method was used to evaluate the cumulative rate of progression to critical illness, and a log-rank test was used to assess differences between groups. HRs were calculated using the Cox regression model. Variables with $P < 0.05$ in the univariate analysis were included in a stepwise Cox proportional hazards regression model. We performed tests for linear trend by entering the median value of each quartile of the NLR as a continuous variable in the models. The Cox proportional hazards model was used to estimate the HRs associated with the NLR for the risk of progression to critical illness with adjustment for pertinent variables. The HRs and 95% CIs of the progression to critical illness in each subgroup were estimated, and their interactions tested. Statistical analyses were conducted using SPSS version 26.0 (IBM Corporation, Armonk) and R version 3.4 (R Foundation). A two-sided P value < 0.05 was considered to indicate statistical significance.

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CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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REFERENCES

1. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

2. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, Ren R, Leung KS, Lau EH, Wong JY, Xing X, Xiang N, Wu Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med. 2020; 382:1199–207. https://doi.org/10.1056/NEJMoa2001316 PMID:31995857

3. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research

Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

4. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

5. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, Xing F, Liu J, Yip CC, Poon RW, Tsoi HW, Lo SK, Chan KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet. 2020; 395:514–23. https://doi.org/10.1016/S0140-6736(20)30154-9 PMID:31986261

6. Lian J, Jin X, Hao S, Cai H, Zhang S, Zheng L, Jia H, Hu J, Gao J, Zhang Y, Zhang X, Yu G, Wang X, et al. Analysis of epidemiological and clinical features in older patients with corona virus disease 2019 (COVID-19) out of Wuhan. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa242 PMID:32211844

7. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

8. Garg S, Kim L, Whitaker M, O'Halloran A, Cummings C, Holstein R, Prill M, Chai SJ, Kirley PD, Alden NB, Kawasaki B, Yousey-Hindes K, Niccolai L, et al. Hospitalization rates and characteristics of patients hospitalized with laboratory-confirmed coronavirus disease 2019 - COVID-NET, 14 states, March 1-30, 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:458–64. https://doi.org/10.15585/mmwr.mm6915e3 PMID:32298251

9. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, Cereda D, Coluccello A, Foti G, Fumagalli R, Iotti G, Latronico N, Lorini L, et al, and COVID-19 Lombardy ICU Network. Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the lombardy region, Italy. JAMA. 2020; 323:1574–81. https://doi.org/10.1001/jama.2020.5394 PMID:32250385

10. Gong J, Ou J, Qiu X, Jie Y, Chen Y, Yuan L, Cao J, Tan M, Xu W, Zheng F, Shi Y, Hu B. A tool to early predict severe corona virus disease 2019 (COVID-19): a multicenter study using the risk nomogram in Wuhan and guangdong, China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa443 PMID:32296824

11. Wynants L, Van Calster B, Collins GS, Riley RD, Heinze G, Schuit E, Bonten MM, Damen JA, Debray TP, De Vos M, Dhiman P, Haller MC, Harhay MO, et al. Prediction models for diagnosis and prognosis of covid-19 infection: systematic review and critical appraisal. BMJ. 2020; 369:m1328. https://doi.org/10.1136/bmj.m1328 PMID:32265220

12. Chen R, Liang W, Jiang M, Guan W, Zhan C, Wang T, Tang C, Sang L, Liu J, Ni Z, Hu Y, Liu L, Shan H, et al, and Medical Treatment Expert Group for COVID-19. Risk factors of fatal outcome in hospitalized subjects with coronavirus disease 2019 from a nationwide analysis in China. Chest. 2020; 158:97–105. https://doi.org/10.1016/j.chest.2020.04.010 PMID:32304772

13. Wang L, He W, Yu X, Hu D, Bao M, Liu H, Zhou J, Jiang H. Coronavirus disease 2019 in elderly patients: characteristics and prognostic factors based on 4-week follow-up. J Infect. 2020; 80:639–45. https://doi.org/10.1016/j.jinf.2020.03.019 PMID:32240670

14. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091. https://doi.org/10.1136/bmj.m1091 PMID:32217556

15. Jia W, Wu J, Jia H, Yang Y, Zhang X, Chen K, Su F. The peripheral blood neutrophil-to-lymphocyte ratio is superior to the lymphocyte-to-monocyte ratio for predicting the long-term survival of triple-negative breast cancer patients. PLoS One. 2015; 10:e0143061. https://doi.org/10.1371/journal.pone.0143061 PMID:26580962

16. Choi JE, Villarreal J, Lasala J, Gottumukkala V, Mehran RJ, Rice D, Yu J, Feng L, Cata JP. Perioperative neutrophil:lymphocyte ratio and postoperative NSAID use as predictors of survival after lung cancer surgery: a retrospective study. Cancer Med. 2015; 4:825–33. https://doi.org/10.1002/cam4.428 PMID:25756351

17. Liu Y, Du X, Chen J, Jin Y, Peng L, Wang HH, Luo M, Chen L, Zhao Y. Neutrophil-to-lymphocyte ratio as an independent risk factor for mortality in hospitalized patients with COVID-19. J Infect. 2020; 81:e6–12. https://doi.org/10.1016/j.jinf.2020.04.002 PMID:32283162

18. Du RH, Liang LR, Yang CQ, Wang W, Cao TZ, Li M, Guo GY, Du J, Zheng CL, Zhu Q, Hu M, Li XY, Peng P, Shi HZ. Predictors of mortality for patients with COVID-19 pneumonia caused by SARS-CoV-2: a prospective cohort study. Eur Respir J. 2020; 55:2000524. https://doi.org/10.1183/13993003.00524-2020 PMID:32269088

19. Liang WH, Guan WJ, Li CC, Li YM, Liang HR, Zhao Y, Liu XQ, Sang L, Chen RC, Tang CL, Wang T, Wang W, He QH, et al. Clinical characteristics and outcomes of hospitalised patients with COVID-19 treated in Hubei (epicentre) and outside Hubei (non-epicentre): a nationwide analysis of China. Eur Respir J. 2020; 55:2000562. https://doi.org/10.1183/13993003.00562-2020 PMID:32269086

20. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, Akdis CA, Gao YD. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. Allergy. 2020; 75:1730–1741. https://doi.org/10.1111/all.14238 PMID:32077115

21. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

22. Opal SM, Girard TD, Ely EW. The immunopathogenesis of sepsis in elderly patients. Clin Infect Dis. 2005 (Suppl 7); 41:S504–12. https://doi.org/10.1086/432007 PMID:16237654

23. Chung M, Bernheim A, Mei X, Zhang N, Huang M, Zeng X, Cui J, Xu W, Yang Y, Fayad ZA, Jacobi A, Li K, Li S, Shan H. CT imaging features of 2019 novel coronavirus (2019-nCoV). Radiology. 2020; 295:202–07. https://doi.org/10.1148/radiol.2020200230 PMID:32017661

24. Zhong Q, Li Z, Shen X, Xu K, Shen Y, Fang Q, Chen F, Liang T. [CT imaging features of patients with different clinical types of coronavirus disease 2019 (COVID-19)]. Zhejiang Da Xue Xue Bao Yi Xue Ban. 2020; 49:0. PMID:32207591

25. Du Y, Tu L, Zhu P, Mu M, Wang R, Yang P, Wang X, Hu C, Ping R, Hu P, Li T, Cao F, Chang C, et al. Clinical features of 85 fatal cases of COVID-19 from Wuhan. A retrospective observational study. Am J Respir Crit Care Med. 2020; 201:1372–79. https://doi.org/10.1164/rccm.202003-0543OC PMID:32242738

26. Marrie TJ, Shariatzadeh MR. Community-acquired pneumonia requiring admission to an intensive care unit: a descriptive study. Medicine (Baltimore). 2007; 86:103–11. https://doi.org/10.1097/MD.0b013e3180421c16 PMID:17435590

27. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang X, Zhang M, Wu S, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest. 2020; 130:2620–29. https://doi.org/10.1172/JCI137244 PMID:32217835

28. Mo P, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, Xiong Y, Cheng Z, Gao S, Liang K, Luo M, Chen T, Song S, et al. Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa270 PMID:32173725

29. Zhang HF, Ge YL, Wang HY, Zhang Q, Li WQ, Chen Y, Chen QC, Jin JJ, Xu J, Zhang S, Xu TT, Zhang X, Yu HL, et al. Neutrophil-to-lymphocyte ratio improves the accuracy and sensitivity of pneumonia severity index in predicting 30-day mortality of CAP patients. Clin Lab. 2019; 65. https://doi.org/10.7754/Clin.Lab.2019.190226 PMID:31625349

30. Zhang F, Ren Y, Fu W, Wang Y, Qian J, Tao C, You C, Yang M. Association between neutrophil to lymphocyte ratio and blood glucose level at admission in patients with spontaneous intracerebral hemorrhage. Sci Rep. 2019; 9:15623. https://doi.org/10.1038/s41598-019-52214-5 PMID:31666624

31. Deng Y, Fan X, Ran Y, Xu X, Lin L, Cui B, Hou L, Zhao T, Wang Y, Su Z, Jiang X, Zhao W, Wang B, Sun C. Prognostic impact of neutrophil-to-lymphocyte ratio in cirrhosis: a propensity score matching analysis with a prespecified cut-point. Liver Int. 2019; 39:2153–63. https://doi.org/10.1111/liv.14211 PMID:31408916

32. Bartlett EK, Flynn JR, Panageas KS, Ferraro RA, Sta Cruz JM, Postow MA, Coit DG, Ariyan CE. High neutrophil-to-lymphocyte ratio (NLR) is associated with treatment failure and death in patients who have melanoma treated with PD-1 inhibitor monotherapy. Cancer. 2020; 126:76–85. https://doi.org/10.1002/cncr.32506 PMID:31584709

33. WHO. Clinical management of severe acute respiratory infection when Novel coronavirus (nCoV) infection is suspected: interim guidance. 2020. https://apps.who.int/iris/handle/10665/330893

34. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19)

outbreak in China: summary of a report of 72 314 cases from the Chinese center for disease control and prevention. JAMA. 2020. [Epub ahead of print].
https://doi.org/10.1001/jama.2020.2648
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Research Paper

Clinical imaging characteristics of inpatients with coronavirus disease-2019 in Heilongjiang Province, China: a retrospective study

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ABSTRACT

Objective: To investigate the clinical, laboratory, and radiological characteristics of patients with coronavirus disease-2019 (COVID-19) in Heilongjiang Province.

Results: Patients in the ICU group were older and their incidence of cardiovascular disease was higher than those in the non-ICU group. Lymphocyte levels were lower and neutrophil and D-dimer levels were higher in the ICU than that in the non-ICU group. Compared to the non-ICU group, the incidence of pulmonary consolidation and ground-glass opacity with consolidation was significantly higher in the ICU group, all lung lobes were more likely to be involved, with higher number of lung lobes and areas surrounding the bronchi. Of the 59 patients with COVID-19 in this group, 15 received mechanical ventilation. All intubated patients involved lung lobes, and a large number of lesions were observed in the area around the bronchial vessels.

Conclusion: Significant differences were observed in clinical symptoms, laboratory tests, and computed tomography features between the ICU and non-ICU groups.

Methods: A total of 59 patients with COVID-19, comprising 44 patients in the intensive care unit (ICU) and 15 in the non-ICU, were retrospectively analyzed. Characteristics of the two groups of patients were compared.

INTRODUCTION

An outbreak of new pneumonia caused by the 2019 novel coronavirus (2019-nCoV) started in Wuhan, China, in December 2019 [1]. In January 2020, Chinese scientists isolated this 2019-nCoV from patients with viral pneumonia, officially naming it as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2]. Since then, the disease has rapidly spread from Wuhan to other regions. In February 2020, the World Health Organization (WHO) named the disease caused by this virus as coronavirus disease 2019 (COVID-19). At the time of this article's submission, some cases have been reported internationally across the six continents.

The COVID-19 pandemic has caused severe illness in infected patients, such as pneumonia and acute respiratory distress syndrome, which even resulted in death. According to the COVID-19 joint study report released by the National Health Commission of the People's Republic of China, about 80% of patients have light and common infection, whereas 13.8% have

severe/critical infections, making them highly at risk for mortality [3]. In addition, prevention and control of severe and critically ill patients are yet to be implemented [3]. Thus, clinicians and radiologists should identify the characteristic imaging manifestations in chest CT findings of critically ill individuals, so that they can perform specific symptomatic treatment at the earliest, prevent complications, and provide organ functional support. Compared to other methods, computed tomography (CT) is the best technique for the early detection of pneumonia. Only a few reports demonstrated the clinical imaging features of severe and critically ill patients during the epidemic in Heilongjiang Province. This study describes the clinical and radiological characteristics and laboratory examination data of 59 patients with COVID-19 and compares between those admitted in the intensive care unit (ICU) and non-ICU departments. Thus, we hope that these current results could be used by clinicians in Heilongjiang Province and worldwide for the treatment plan of COVID-19.

RESULTS

A total of 59 patients confirmed with COVID-19 in Heilongjiang Province were included in this study. The general clinical data of patients are shown in Table 1. The median age was 64.0 (IQR, 56–72) years. The most common complication in the patient group was cardiovascular disease (44%), followed hypertension (42%) and diabetes (15%), and the rarest complication was chronic obstructive disease (3%), followed by malignancy (2%) and chronic liver disease (2%). Compared to non-ICU patients, ICU patients were older (median age: 67 *vs.* 56); $P = 0.037$) and more likely at risk for cardiovascular diseases (52% *vs.* 20%; $P = 0.030$). The most common clinical symptoms in this study were fever (41/59, 69%), cough (30/59, 51%), and muscle soreness (15/59, 25%), whereas the less common were dyspnea (14/59, 24%), headache (8/59, 13%), abdominal pain, diarrhea (5/59, 8%), and nausea (3/59, 5%). However, compared to non-ICU patients, the incidence of muscle soreness in the ICU patients was reduced (18% *vs.* 47%; $P = 0.042$).

Laboratory examination results of 59 patients are summarized in Table 2. White blood cell count ($<4 \times 10^9$/L; 11/59, 19%) and lymphocyte count ($<1.0 \times 10^9$/L; 26/59, 44%) were low in some patients. Compared to non-ICU patients, ICU patients are more likely to have lymphopenia (52% *vs.* 20%; $P = 0.003$), with higher neutrophil and D-dimer levels (median: 3.5 [IQR, 2.6–5.2] *vs.* median 1.7 [IQR, 0.8–3.1], $P = 0.003$; median 364.6 [IQR, 3.5–1475.0] *vs.* median 0.5 [IQR, 0.4–6.5], $P = 0.000$, respectively) and lower hemoglobin levels (median, 100.5 [IQR, 86.0–115.0] *vs.* median, 128.0 [IQR, 122.0–136.0], $P < 0.001$).

All patients (59/59; 100%) showed abnormal CT findings (Table 3). The main features of the imaging examination were ground-glass opacity (58/59; 98%; Figure 1A), consolidation (37/59; 63%), and ground-glass opacity combined with consolidation (36/59; 61%; Figure 1B). Compared to non-ICU patients, the incidence of consolidation and ground-glass opacity combined with consolidation in ICU patients was higher (73% *vs.* 33%, $P = 0.006$; 70% *vs.* 33%, $P = 0.011$, respectively). Furthermore, 40/59 (68%) patients showed involvement of all lung lobes in the ICU group (Figure 1C) as compared to the non-ICU patients, whereas the incidence of all lung lobes (75% *vs.* 47%, $P = 0.043$) and the number of lung lobes were higher in patients with ICU (median, 5 [IQR, 5–5] *vs.* median, 4 [IQR, 2–5], $P = 0.012$). Among 59 patients with COVID-19, 43 (73%) were multifocal, 15 (25%) were diffuse, and only 1 (2%) was focal. A significant difference was detected in the degree of lung involvement between ICU and non-ICU patients ($P = 0.032$). Furthermore, 23 (39%) patients had abnormal density shadows around the bronchi: 21/44 (48%) ICU patients and 2/15 (13%) non-ICU patients. The incidence of bronchovascular involvement in ICU patients was significantly higher than that in non-ICU patients (48% *vs.* 13%, $P = 0.040$), which might be observed by breathing difficulty and need for mechanical ventilation (Figure 1D). Unilateral or bilateral pleural effusion occurred in 7/59 (12%) patients: 6 in the ICU group (6/44, 14%) and 1 in the non-ICU group (1/15, 7%). In addition, mediastinal lymphadenopathy (short axis, >1 cm) was observed in 13 of 59 patients (22%), fibrous cord shadow in 22 (37%), and arterial plaque in 32 (54%).

A total of 15 (25%) patients were intubated with respiratory failure. All of them (100%) had ground-glass opacity, showed bilateral lung involvement, and involved more than three lung lobes. Compared to the non-mechanically ventilated patients, these patients requiring mechanical ventilation were more likely to have abnormal lung changes in the area around the bronchi (53% *vs.* 34%) and showed diffuse distribution (47% *vs.* 18%).

DISCUSSION

COVID-19 is a new viral outbreak that may have a profound impact on public health. With the increased number of confirmed cases, the number of severe and critical cases in Heilongjiang Province is also continuously increasing. This might be caused by lung tissue inflammation, which in turn, causes organ dysfunction and is even life-threatening. In addition, patients who are severely/critically ill have poor prognosis and higher mortality than non-critically ill

Table 1. Demographics and baseline characteristics of two groups of patients infected with 2019-nCoV.

	All patients (n=59)	ICU care (n=44)	No ICU care (n=15)	*P* value
Characteristics				
Age (y)	64.0(56.0-72.0)	66.5(57.3-75.8)	56.0(50.0-68.0)	0.037
Gender				0.552
Male	29(49%)	23(52%)	6(40%)	
Female	30(51%)	21(48%)	9(60%)	
Exposure history				0.516
Contact with infected patients	42(71%)	30(68%)	12(80%)	
Unknown history	17 (29%)	14(32%)	3(20%)	
Any comorbidity				
Diabetes	9(15%)	6(14%)	3(20%)	0.680
Hypertension	25(42%)	20(45%)	5(33%)	0.412
Cardiovascular disease	26(44%)	23(52%)	3(20%)	0.030
COPD	2(3%)	1(2%)	1(7%)	0.447
Malignancy	1(2%)	1(2%)	0(0%)	--
Chronic liver disease	1(2%)	0(0%)	1(7%)	--
Signs and symptoms				
Fever	41(69%)	31(70%)	10(67%)	0.785
Highest temperature, °C				0.412
<37.3	18(31%)	14(32%)	4(27%)	
37.3–38.0	25(42%)	16(36%)	9(60%)	
38.1–39.0	15(25%)	13(30%)	2(13%)	
>39.0	1(2%)	1(2%)	0(0%)	
Cough	30(51%)	20(45%)	10(67%)	0.205
Myalgia or fatigue	15(25%)	8(18%)	7(47%)	0.042
Headache	8 14%)	4(9%)	4(27%)	0.184
Diarrhoea, bellyache	5(8%)	4(9%)	1(7%)	0.624
Dyspnoea	14(24%)	9(20%)	5(33%)	0.316
Nausea	3(5%)	1(2%)	2(13%)	0.156

Data are median (IQR), n (%), or n/N (%), where N is the total number of patients with available data. *P* values comparing Group1 and Group2 are from χ^2 test, Fisher's exact test, or Mann-Whitney U test. 2019-nCoV=2019 novel coronavirus. COPD=Chronic obstructive pulmonary disease.

Table 2. Laboratory findings of two groups of patients infected with 2019-nCoV.

Laboratory Findings	All patients (n=59)	ICU care (n=44)	No ICU care (n=15)	*P* value
White blood cell count($\times 10^9$/L)	5.5(4.3-7.1)	5.2(4.1-7.0)	5.8(4.6-7.0)	0.334
<4	11(19%)	9(20%)	2(13%)	0.894
4-10	42(71%)	30(68%)	12(80%)	
>10	6(10%)	5(11%)	1(7%)	
Neutrophil count($\times 10^9$/L)	3.2(1.9-4.8)	3.5(2.6-5.2)	1.7(0.8-3.1)	0.003
Lymphocyte count($\times 10^9$/L)	1.1(0.6-1.5)	0.9(0.6-1.3)	1.6(0.9-2.3)	0.004
<1.0	26(44%)	23(52%)	3(20%)	0.030
≥1.0	33(56%)	21(48%)	12(80%)	
Haemoglobin, g/L	104.0(92.0-122.0)	100.5(86.0-115.0)	128.0(122.0-136.0)	0.000
Platelet count($\times 10^9$/L)	189.0(145.0-260.0)	194.5(142.0-264.5)	189.0(152.0-255.0)	0.734
<100	11(19%)	9(20%)	2(13%)	0.712
≥100	48(81%)	35(80%)	13(87%)	
Prothrombin time, s	12.4(12.0-13.3)	12.6(12.0-13.4)	12.0(11.9-13.0)	0.458
Activated partial thromboplastin time, s	30.9(28.0-33.3)	31.0(27.0-33.9)	30.5(29.0-31.8)	0.651

D-dimer, mg/L	6.1(1.5-1090.0)	364.6(3.5-1475.0)	0.5(0.4-6.5)	0.000
C-reactive protein, mg/L	8.4(2.0-30.9)	9.9(0.3-180.7)	8.0(0.2-77.9)	0.807
Alanine aminotransferase, U/L	37.6(30.2-45.0)	37.8(25.9-46.7)	36.7(34.4-40.7)	0.862
Aspartate aminotransferase, U/L	26.5(21.2-33.3)	26.5(19.3-35.0)	26.1(23.8-33.3)	0.708
≤40	51(86%)	36(82%)	15(100%)	0.100
>40	8(14%)	8(18%)	0(0%)	
Creatinine, μmol/L	57.1(44.7-89.9)	55.7(42.0-83.0)	89.9(57.0-133.0)	0.008
≤133	53(90%)	41(93%)	12(80%)	0.165
>133	6(10%)	3(7%)	3(20%)	
Creatine kinase, U/L	116.0(34.6-175.3)	130.1(34.8-200.0)	113.9(31.5-167.7)	0.676
≤185	45(76%)	32(73%)	13(87%)	0.483
>185	14(24%)	12(27%)	2(13%)	

Data are median (IQR) or n/N (%), where N is the total number of patients with available data. p values comparing Group1 and Group2 are from χ^2, Fisher's exact test, or Mann-Whitney U test. 2019-nCoV=2019 novel coronavirus.

Table 3. CT diagnosis characteristics of two groups of patients infected with 2019-nCoV.

Imaging Findings	All patients (n=59)	ICU care (n=44)	No ICU care (n=15)	*P* value
Parenchymal opacities				
Consolidation	37(63%)	32(73%)	5(33%)	0.006
GGO	58(98%)	43(98%)	15(100%)	0.746
GGO and consolidation	36(61%)	31(70%)	5(33%)	0.011
Reticular opacities	13(22%)	7(16%)	6(40%)	0.073
Nodular opacities	11(19%)	8(18%)	3(20%)	0.574
Laterality				0.265
Bilateral	4(7%)	2(5%)	2(13%)	
Unilateral	55(93%)	42(95%)	13(87%)	
Involvement range of lung lobes				
All lung lobe	40(68%)	33(75%)	7(47%)	0.043
Right upper lobe	51(86%)	38(86%)	7(47%)	0.673
Right middle lobe	49(83%)	39(89%)	10(67%)	0.104
Right lower lobe	54(92%)	42(95%)	12(80%)	0.099
Left upper lobe	51(86%)	39(89%)	12(80%)	0.407
Left lower lobe	52(88%)	41(93%)	11(73%)	0.062
Number of lung lobes, mean	5(4-5)	5(5-5)	4(2-5)	0.012
Distribution				
Central and peripheral	9(15%)	8(18%)	1(7%)	0.424
Central	12(20%)	11(25%)	1(7%)	0.160
Peripheral	53(90%)	39(89%)	14(93%)	0.518
Peribronchovascular	23(39%)	21(48%)	2(13%)	0.040
Extent				0.032
Single shot	1(2%)	0(0%)	1(7%)	
Multiple	43(73%)	30(68%)	13(87%)	
Diffuse	15(25%)	14(32%)	1(7%)	
Pleural effusion	6(10%)	3(7%)	3(20%)	0.165
Arterial plaque	22(37%)	15(34%)	7(47%)	0.384
Fiber rope	32(54%)	27(61%)	5(33%)	0.060
Mediastinal lymphadenopathy	13(22%)	10(23%)	3(20%)	0.569

Data is n/N (%), where N is the total number of patients with available data. Abbreviations: CT, computed tomography; GGO, ground-glass opacity.

ones [6, 7]. A recent assessment showed that the fatality rate of severe pneumonia is 30–50%, leading to severe complications and increasing the medical burden [8]. Thus, early identification of such cases based on changes in chest radiography and clinical features is crucial. In the present study, clinical and imaging characteristics of patients with COVID-19 in the ICU group were determined by comparing the ICU and non-ICU patients.

The most common clinical symptoms in this group of patients were fever and cough. We found that the ICU group was older and more likely to have cardiovascular disease than the non-ICU group. Moreover, older people or people with poor health conditions were found to have a worsening pneumonia, which might be due to the weakened immune system [9]. According to a study report on patients with COVID 19 in Wuhan [10], the probability of all patients with hypertension and cardiovascular disease is 15% and 15%, whereas the corresponding incidence in patients with COVID 19 in Heilongjiang Province is 42% and 44%, which may be attributed to the specific geographical environment of Heilongjiang Province, resulting in a high incidence of cardiovascular diseases. Studies on SARS-CoV and Middle East Respiratory Syndrome (MERS)-CoV infections demonstrated that the risk of exacerbation markedly increases with age and presence of underlying diseases [11–13], which was consistent with the conclusions of this study. The difference in the male-to-female ratio was not significant between the two groups, indicating that gender is not a high-risk cause of disease severity, which is consistent with that of a recent report [14]. Compared to the ICU group, the incidence of muscle soreness was significantly higher in the non-ICU group. This clinical symptom is rarely observed in other related studies and may be related to regional environmental characteristics. Taken together, these clinical manifestations can help clinicians determine the disease severity in clinical practice. Other

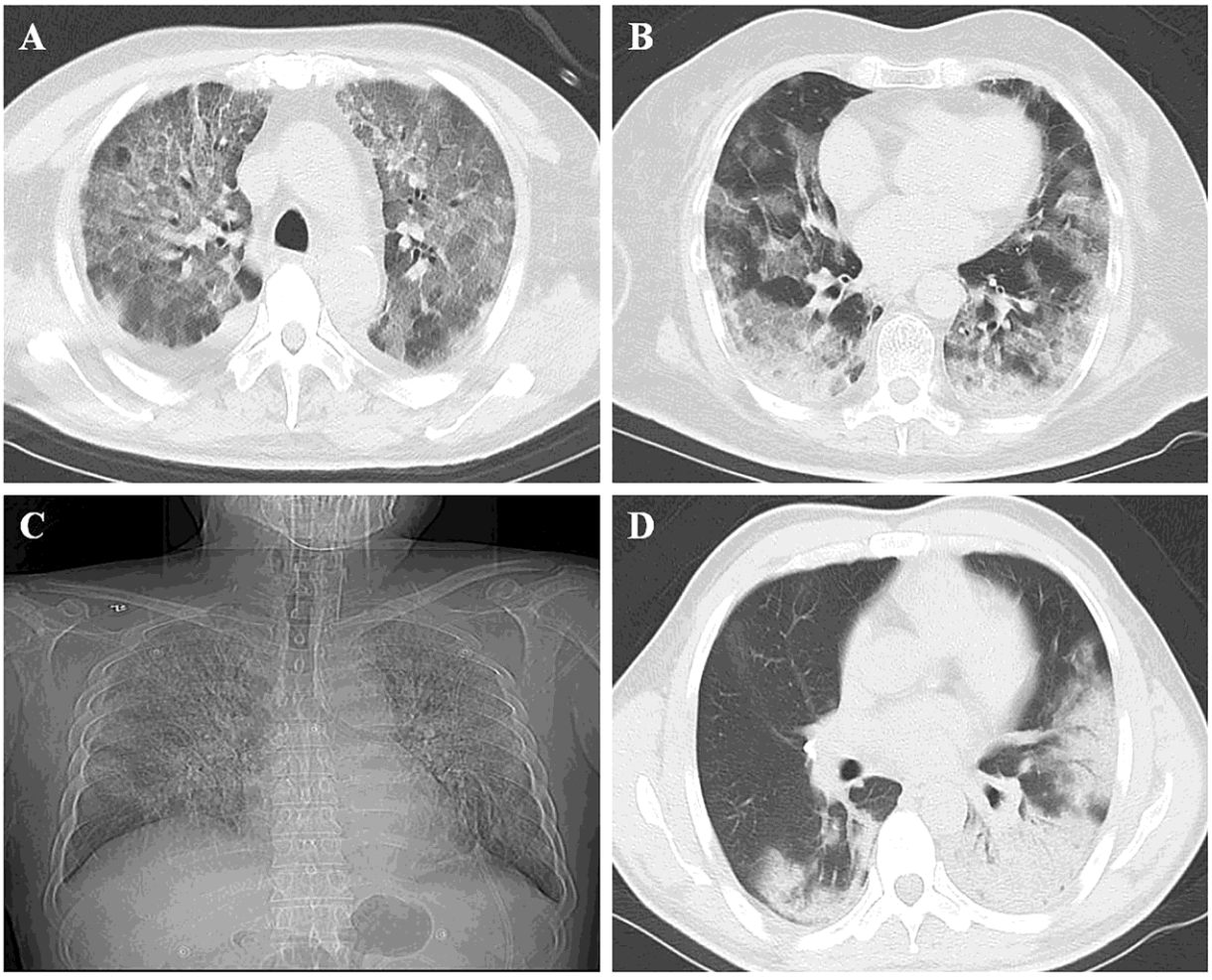

Figure 1. Chest imaging of patients with COVID-19. (**A**) Ground-glass opacity; (**B**) Lesion with ground-glass opacity and consolidation; (**C**) Lesion involving all lung lobes of both lungs; (**D**) Lesion involving the surrounding area of the bronchial blood vessel.

symptoms in our patients with COVID-19 were similar to that of other coronavirus infections, including dyspnea, headache, abdominal pain, diarrhea, and nausea. For example, SARS and MERS may belong to the same attributed infection and also indicate that the SARS-CoV-2 target cells are located in the lower respiratory tract [15–17].

The present study identified multiple laboratory index differences between non-ICU groups and ICU groups, including lymphocyte, neutrophil, and D-dimer levels. Compared to the non-ICU group, the ICU group is prone to lymphopenia, which is consistent with the results of the latest research report of patients with COVID-19 in Wuhan and China [10, 18]. Lymphopenia in the ICU group indicates that a large number of immune cells are consumed and the immune function is suppressed, demonstrating that lymphocyte damage may be the key to the deterioration of the patient's condition; therefore, decreased lymphocyte count could be a critical indicator of disease severity [19]. Increased neutrophil and D-dimer levels in patients in the ICU group may be related to cytokine storms caused by the viral invasion, which is supported by recent studies [9, 20]. Notably, patients with high D-dimer levels for the first time are predictive of poor prognosis [20], which is consistent with the opinion of this study.

From a broad perspective, CT manifestations of COVID-19 pneumonia are similar to that of other viral pneumonia. Imaging findings of viral pneumonia include reticular pattern and patchy or diffuse ground-glass opacity, with or without consolidation [21]. In influenza pneumonia, lobular septal thickening and grid-like density shadows are frequently observed, whereas pleural effusion is rare [21]. Despite similarities, some of our patients' imaging findings are different from those of the traditional seasonal flu.

In this study, all patients with COVID-19 had abnormal chest CT findings. Additionally, ground-glass opacity (98%) and consolidation (63%) are the most common imaging findings in the current study, which is consistent with the results of the recent COVID-19 studies [22]. This phenomenon may be related to exudative inflammation caused by alveolar and interstitial edema of the lung due to viral invasion, and CT is mainly manifested as ground-glass opacity [23]. An autopsy report of patients with COVID-19 pneumonia deaths shows that the ground-glass opacity corresponds to the gray-white alveolar lesions observed by the naked eye, suggesting that the virus mainly causes inflammatory reactions characterized by deep airway and alveolar damage [24]. Herein, we found that compared to the non-ICU group, the incidence of consolidation and ground-glass opacity combined with consolidation in patients in the ICU group was higher (P = 0.006; P = 0.011), indicating that the alveoli of critically ill patients were filled with inflammatory exudates. This means that the virus has spread to the respiratory tract, leading to necrotic bronchitis and diffuse alveolar damage [25, 26], which is consistent with the results of recently published studies [27–29]. Among the 59 (68%) patients, 40 displayed imaging abnormalities involving all lung lobes (5) as compared to 7/15 (47%) of non-ICU patients, whereas 33/44 (75%) of all ICU patients were involved; the difference between the two groups was statistically significant (P = 0.043). In addition, we found that the degree of involvement of lung lesions was statistically significant between the two groups (P = 0.032). Chest imaging features may help the early prediction of the patients' clinical development early.

In this group of patients, 15 needed mechanical ventilation. Compared to non-mechanical ventilation patients, CT abnormalities in the lungs of patients requiring mechanical ventilation were primarily distributed around the bronchial blood vessels, and diffuse distribution was likely to occur, making patients prone to dyspnea. Some other studies demonstrated that the distribution of abnormal lesions during CT examination may be the decisive factor for the clinical course of patients with COVID-19 [22, 30]. Other imaging features in this study included bilateral lung involvement in 93% of patients, and majority of them (90%) had lung lesions in the peripheral area without emphysema or pulmonary nodules; these imaging abnormalities and distribution patterns are consistent the previously published results [31, 32]. Among the patients in this study, only 7 (12%) had pleural effusion, including 6 (14%) in the ICU group and 1 (7%) in the non-ICU group. Furthermore, pleural effusion is a rare imaging manifestation in patients with COVID-19, and the incidence rate in the ICU group is higher than that in the non-ICU group, which is consistent with the results of Junhua et al.'s study [33].

Nevertheless, this study has some limitations. (1) None of the patients underwent lung biopsy or autopsy, which might have established a correlation between imaging and histopathology. (2) The sample size of the non-ICU group is relatively small. Collecting standardized data for larger populations will help explore clinical manifestations and high-risk factors. (3) As most patients are still in the hospital at the time of submission of this manuscript, risk factors for poor prognosis were not assessed.

CONCLUSIONS

In summary, existing cardiovascular disease, fever, and cough in elderly patients with COVID-19 may worsen

the condition. Lymphopenia and elevated neutrophil and D-dimer levels are also indicators of COVID-19 disease progression. In addition, imaging findings of patients with severe COVID-19 mainly include consolidation and ground-glass opacity combined with consolidation, which putatively involves all lung lobes and the area around the bronchi. Since several patients are currently in the critical stage, we hope that the results of this study would be beneficial for the disease control, diagnosis, treatment, and prognosis in Heilongjiang Province and worldwide and even reduce the mortality rate.

MATERIALS AND METHODS

Study population

The study has been approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and is in accordance with the Helsinki Declaration. According to the COVID-19 pneumonia diagnostic criteria for the diagnosis and treatment of new coronavirus-caused pneumonia (trial version 6) issued by the National Health Commission of the People's Republic of China [4], the inclusion criteria were as follows: (1) real-time fluorescent reverse transcription-polymerase chain reaction (RT-PCR) for detection of positive cDNA of SARS-CoV-2; (2) untreated newly diagnosed patients; (3) patients with complete clinical data; and (4) all patients who underwent at least one CT scan. Exclusion criteria were as follows: (1) treated non-newly diagnosed patients and (2) missing clinical data. This study included a total of 76 patients confirmed with COVID-19 between February and March 2020, and 59 of them met the above criteria. The cohort was divided into the ICU (n = 44) and non-ICU groups (n = 15). Clinical data of all patients were evaluated: background information such as gender and age and clinical symptoms such as fever, cough, and underlying diseases (hypertension, diabetes, cardiovascular disease, and chronic obstructive pulmonary disease). Laboratory examination results upon admission, including white blood cells, lymphocytes, neutrophils, D-dimer, and C-reactive protein levels, as well as imaging data, were collected.

Image analysis

All CT images were analyzed and diagnosed by two radiologists trained for novel coronavirus. Both radiologists have >5 years of diagnostic experience. Two doctors independently diagnosed all patient images and reached a consensus. In case of disagreement between the two radiologists, a third trained radiologist with >10 years of diagnostic experience was consulted to reach a consensus. Imaging features (ground-glass opacity, consolidation, reticular pattern, and nodular opacity), lesion distribution (unilateral/bilateral, upper/middle/lower lobe, and central/peripheral/bronchial blood vessel surrounding), and degree of involvement (focal/multifocal/diffuse and number of lung lobes) were all abnormal. Radiographic images and CT scans using descriptors were defined using the Fleischner Society Naming Committee [5]. Ground-glass opacity is defined as a hazy area showing increased lung opacity with indistinct pulmonary vessel margins on a radiograph but with preserved bronchial and vascular margins on CT. Consolidation is defined as a homogeneous increase in parenchymal attenuation that obscures vessel margins and airway walls. The reticular pattern is defined as small linear opacities forming a net pattern. Nodular opacity is defined as a well- or poorly defined rounded opacity, measuring up to 3 cm in diameter. Lesion distribution features include unilateral/bilateral and upper/middle/lower lobes. The extent of lesion involvement was divided into focality, multifocality, and diffuse. Focality is defined as an abnormal single lesion, whereas multifocality is defined as the presence of more than one lesions; if it is diffusely distributed, it involves one or both lungs. Moreover, whether the lesion occurs centrally (<4 cm from the hilum) or peripherally or involves the bronchi should be determined. The presence of pleural effusion, laterality, and any other lung findings such as mediastinal lymphadenopathy was also noted.

Statistical analysis

The SPSS 19.0 statistical software was used for analysis. Continuous variables were expressed as median (interquartile ratio [IQR]) and compared using the Mann–Whitney U test. Categorical variables were expressed as number of cases (n) and percentage/rate (%); χ^2 test or Fisher's exact test was used to compare ICU and non-ICU groups. $P < 0.05$ was considered statistically significant.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. World Health Organization. WHO/Novel Coronavirus-China. 2020. https://www.who.int/csr/don/12-january-2020-novel-coronavirus-china/en/

2. Gorbalenya AE. Severe acute respiratory syndrome-related coronavirus–The species and its viruses, a statement of the Coronavirus Study Group. BioRxiv. 2020.

3. National Health Commission of the People's Republic of China, World Health Organization. China-WHO new coronavirus pneumonia (COVID-19) joint inspection report. 2020. https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf

4. National Health Commission of the People's Republic of China. New coronavirus pneumonia diagnosis and treatment plan (trial version 6). 2020.

5. Hansell DM, Bankier AA, MacMahon H, McLoud TC, Müller NL, Remy J. Fleischner society: glossary of terms for thoracic imaging. Radiology. 2008; 246:697–722. https://doi.org/10.1148/radiol.2462070712 PMID:18195376

6. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. Lancet. 2020; 395:470–73. https://doi.org/10.1016/S0140-6736(20)30185-9 PMID:31986257

7. Bernheim A, Mei X, Huang M, Yang Y, Fayad ZA, Zhang N, Diao K, Lin B, Zhu X, Li K, Li S, Shan H, Jacobi A, Chung M. Chest CT findings in coronavirus disease-19 (COVID-19): relationship to duration of infection. Radiology. 2020; 295:200463. https://doi.org/10.1148/radiol.2020200463 PMID:32077789

8. Wu P, Hao X, Lau EH, Wong JY, Leung KS, Wu JT, Cowling BJ, Leung GM. Real-time tentative assessment of the epidemiological characteristics of novel coronavirus infections in Wuhan, China, as at 22 january 2020. Euro Surveill. 2020; 25:2000044. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000044 PMID:31992388

9. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

10. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

11. Cowling BJ, Muller MP, Wong IO, Ho LM, Lo SV, Tsang T, Lam TH, Louie M, Leung GM. Clinical prognostic rules for severe acute respiratory syndrome in low- and high-resource settings. Arch Intern Med. 2006; 166:1505–11. https://doi.org/10.1001/archinte.166.14.1505 PMID:16864761

12. Lau EH, Hsiung CA, Cowling BJ, Chen CH, Ho LM, Tsang T, Chang CW, Donnelly CA, Leung GM. A comparative epidemiologic analysis of SARS in Hong Kong, Beijing and Taiwan. BMC Infect Dis. 2010; 10:50. https://doi.org/10.1186/1471-2334-10-50 PMID:20205928

13. Hui DS, Azhar EI, Kim YJ, Memish ZA, Oh MD, Zumla A. Middle east respiratory syndrome coronavirus: risk factors and determinants of primary, household, and nosocomial transmission. Lancet Infect Dis. 2018; 18:e217–27. https://doi.org/10.1016/S1473-3099(18)30127-0 PMID:29680581

14. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

15. Lee N, Hui D, Wu A, Chan P, Cameron P, Joynt GM, Ahuja A, Yung MY, Leung CB, To KF, Lui SF, Szeto CC, Chung S, Sung JJ. A major outbreak of severe acute respiratory syndrome in hong kong. N Engl J Med. 2003; 348:1986–94. https://doi.org/10.1056/NEJMoa030685 PMID:12682352

16. Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, Flemban H, Al-Nassir WN, Balkhy HH, Al-Hakeem RF, Makhdoom HQ, Zumla AI, Memish ZA. Epidemiological, demographic, and clinical characteristics of 47 cases of middle east respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. Lancet Infect Dis. 2013; 13:752–61. https://doi.org/10.1016/S1473-3099(13)70204-4 PMID:23891402

17. Müller NL, Ooi GC, Khong PL, Zhou LJ, Tsang KW, Nicolaou S. High-resolution CT findings of severe acute respiratory syndrome at presentation and after admission. AJR Am J Roentgenol. 2004; 182:39–44. https://doi.org/10.2214/ajr.182.1.1820039 PMID:14684509

18. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

19. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med. 2020; 8:420–22. https://doi.org/10.1016/S2213-2600(20)30076-X PMID:32085846

20. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

21. Kim EA, Lee KS, Primack SL, Yoon HK, Byun HS, Kim TS, Suh GY, Kwon OJ, Han J. Viral pneumonias in adults: radiologic and pathologic findings. Radiographics. 2002; 22:S137–49. https://doi.org/10.1148/radiographics.22.suppl_1.g02oc15s137 PMID:12376607

22. Li K, Wu J, Wu F, Guo D, Chen L, Fang Z, Li C. The clinical and chest CT features associated with severe and critical COVID-19 pneumonia. Invest Radiol. 2020; 55:327–31. https://doi.org/10.1097/RLI.0000000000000672 PMID:32118615

23. Ketai LH. Conventional wisdom: unconventional virus. AJR Am J Roentgenol. 2009; 193:1486–87. https://doi.org/10.2214/AJR.09.3758 PMID:19933637

24. Liu Q, Wang R, Qu G. Anatomy of a new coronavirus pneumonia death corpse system. J Forensic Med. 2020; 36:19–21.

25. Koo HJ, Lim S, Choe J, Choi SH, Sung H, Do KH. Radiographic and CT features of viral pneumonia. Radiographics. 2018; 38:719–39. https://doi.org/10.1148/rg.2018170048 PMID:29757717

26. Franquet T. Imaging of pulmonary viral pneumonia. Radiology. 2011; 260:18–39. https://doi.org/10.1148/radiol.11092149 PMID:21697307

27. Song F, Shi N, Shan F, Zhang Z, Shen J, Lu H, Ling Y, Jiang Y, Shi Y. Emerging 2019 novel coronavirus (2019-nCoV) pneumonia. Radiology. 2020; 295:210–17. https://doi.org/10.1148/radiol.2020200274 PMID:32027573

28. Pan F, Ye T, Sun P, Gui S, Liang B, Li L, Zheng D, Wang J, Hesketh RL, Yang L, Zheng C. Time course of lung changes at chest CT during recovery from coronavirus disease 2019 (COVID-19). Radiology. 2020; 295:715–21. https://doi.org/10.1148/radiol.2020200370 PMID:32053470

29. Kanne JP. Chest CT findings in 2019 novel coronavirus (2019-nCoV) infections from Wuhan, China: key points for the radiologist. Radiology. 2020; 295:16–17. https://doi.org/10.1148/radiol.2020200241 PMID:32017662

30. Wu J, Wu X, Zeng W, Guo D, Fang Z, Chen L, Huang H, Li C. Chest CT Findings in Patients With Coronavirus Disease 2019 and Its Relationship With Clinical Features. Invest Radiol. 2020; 55:257–261. https://doi.org/10.1097/RLI.0000000000000670 PMID:32091414

31. Xu X, Yu C, Qu J, Zhang L, Jiang S, Huang D, Chen B, Zhang Z, Guan W, Ling Z, Jiang R, Hu T, Ding Y, et al. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2. Eur J Nucl Med Mol Imaging. 2020; 47:1275–80. https://doi.org/10.1007/s00259-020-04735-9 PMID:32107577

32. Chung M, Bernheim A, Mei X, Zhang N, Huang M, Zeng X, Cui J, Xu W, Yang Y, Fayad ZA, Jacobi A, Li K, Li S, Shan H. CT imaging features of 2019 novel coronavirus (2019-nCoV). Radiology. 2020; 295:202–07. https://doi.org/10.1148/radiol.2020200230 PMID:32017661

33. Cheng K, Wei M, Sheng C, Chen D, Xiong W, Zhou X, Zhang D, Zheng J. [Analysis of clinical characteristics of 463 patients with common and severe new coronavirus pneumonia]. Shanghai Medical Journal=Shang Hai Yi Xue. 2020.

Research Paper

Risk of death by age and gender from CoVID-19 in Peru, March-May, 2020

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ABSTRACT

Peru implemented strict social distancing measures during the early phase of the epidemic and is now experiencing one of the largest CoVID-19 epidemics in Latin America. Estimates of disease severity are an essential indicator to inform policy decisions about the intensity and duration of interventions needed to mitigate the outbreak. Here we derive delay-adjusted case fatality risks (aCFR) of CoVID-19 in a middle-income country in South America.

We utilize government-reported time series of CoVID-19 cases and deaths in Peru stratified by age group and gender.

As of May 25, 2020, we estimate the aCFR for men and women at 10.8% (95%CrI: 10.5-11.1%) and 6.5% (95%CrI: 6.2-6.8%), respectively, whereas the overall aCFR was estimated at 9.1% (95%CrI: 8.9-9.3%). Our results show that senior individuals have been the most severely affected by CoVID-19, particularly men, with an aCFR of nearly 60% for those aged 80- years. We also found that men have a significantly higher cumulative morbidity ratio across most age groups (proportion test, p-value< 0.001), with the exception of those aged 0-9 years.

The ongoing COVID-19 pandemic is generating a substantial mortality burden in Peru. Senior individuals, especially those older than 70 years, are being disproportionately affected by the COVID-19 pandemic.

INTRODUCTION

As of May 25, 2020, more than 5.5 million CoVID-19 cases and about340,000 deaths have been reported from almost every country and territory around the globe [1, 2]. The ongoing CoVID-19 pandemic has imposed a substantial burden on health systems, economies, and societies globally, and there are strong indicators pointing to a disproportionate impact on low- and middle-income countries [3–5]. Since its initial outbreak in China, the world has tracked the CoVID-19 pandemic proliferating across Europe and Asia, and later seeding hotspots in

North America, the Middle East, and more recently in Latin America [6]. Brazil reported its first case on February 26, 2020 [7]. Neighboring countries started to report CoVID-19 cases in subsequent days; South America has registered more than 600,000 cases and 30,600 deaths as of May 24, 2020 [1]. Although many South American countries imposed strict control measures, including travel bans, school closures, and lockdowns early in the epidemic, the magnitude of their epidemics now rival those observed in European hotspots, with CoVID-19 cases and death counts increasing rapidly in the region [1, 5]. Other factors, including high poverty rates, informal economies, frail healthcare systems, insufficient medical supplies as well as inadequate water, sanitation, and hygiene infrastructure further exacerbate the health and socioeconomic impacts of the CoVID-19 pandemic [5, 8–10]. Governments in South America are now facing the social and economic consequences from SARS-COV-2 containment measures, while struggling to contain the rapidly expanding outbreaks of the deadly virus [9].

Peru, a country of about 30 million people, is experiencing one of the largest CoVID-19 epidemics in Latin America. With a rapidly rising case tally, Peru has reported almost 129,148 cases and 7660 deaths as of May 25, 2020 [11]. The majority (63%) of CoVID-19 cases have been confirmed in Lima, the capital of Peru [11]. The government of Peru initiated social distancing measures soon after the confirmation of the first imported case in Peru on March 6, 2020 [12]. The initial epidemic control measures included school closures on March 11, 2020 followed by the suspension of large gatherings and flights from Europe and Asia the next day. Subsequently the government declared a national emergency and closed its borders on March 16, 2020 [13]. Despite these forthcoming and swift control measures, untraced community transmission was reported by March 17, 2020, forcing the implementation of a night time curfew as of March 18, 2020 [13].

Estimates of the reproduction number from the early stage of the epidemic in Peru (March 2020) showed sustained transmission in Lima with a reproduction number R estimated at 2.3 (95% CI: 2.0, 2.5) [14]. Moreover, the 20-days ahead forecast for Lima suggested that the prompt social distancing measures had significantly slowed down the initial spread of the virus in the region [14]. Despite the implementation of non-pharmaceutical interventions in Peru, case and death counts have continued to rise rapidly. The crude case fatality risk (CFR), defined as the number of cumulative deaths and cases as of May 25, 2020, in Peru is estimated at 5.9%, which is in good agreement with the global crude CFR average of 6.3% [15]. Statistical analyses and mathematical models using data from Peru suggest that under current epidemic growth trends, the number of CoVID-19 infected individuals could surpass the country's healthcare system capacity [16].

The clinical spectrum of CoVID-19 ranges from asymptomatic cases to clinical conditions characterized by respiratory failure, to multiorgan and systemic manifestations which can cause death [17–19]. The SARS-CoV-2 virus is more likely to generate severe disease among individuals ≥60 years of age, especially those with preexisting medical conditions that include heart disease, lung disease, diabetes or cancer [20]. Further, CoVID-19 associated deaths occur more frequently (about 80% of total deaths) in persons aged ≥65 years based on data from the USA, and consistent with data from China indicating that >80% CoVID-19 deaths occur among persons aged ≥60 years [21]. Moreover, a higher crude fatality risk has been reported among men (2.8% for men versus 1.7% for women) in China [22]. Age adjusted CFR estimates from Peru can be useful to gauge the mortality impact of the pandemic and assess whether the severity patterns are consistent in the South America, a region with fragmented health systems, vast inequality, and high poverty rates.

CFR is a key epidemiological metric that quantifies the severity of an epidemic [23], aiding public health officials assess the type and intensity of interventions that need to be implemented to mitigate its impact [24]. However, it becomes challenging to estimate CFR during an epidemic as CFR estimates are sensitive to right censoring of the data that occurs because of the time lag between the symptoms onset and death [25–27]. Moreover, under-reporting of cases because mild or asymptomatic cases can go undetected by disease surveillance systems also overestimates CFR [25, 28], while CFR estimates by subgroup are less prone to sampling bias and help identify the most vulnerable subpopulations. For comparison, the infection fatality risk (IFR) is calculated by the ratio of cumulative deaths over the cumulative number of infected individuals.

Given the importance of timely CFR estimates for public health decision making, we provide real-time estimates of adjusted age-specific CFR during the CoVID-19 epidemic in Peru, through May 25, 2020 to assess the pandemic's severity variation in this southern hemisphere setting, which helps pinpoint the most vulnerable segments of the population and tailor public health interventions.

RESULTS

As of May 25, a total of 129,148 cases and 7,660 deaths due to CoVID-19 have been reported by the Ministry of

Table 1. Distribution of the cases by sex and age groups, as of May 25, 2020.

Age group	Men				Women			
	Cases (%)	Deaths (%)	cCFR (%)	Mortality per 100,000 population	Cases (%)	Deaths (%)	cCFR(%)	Mortality per 100,000 population
All	78264 (100)	5508 (100)	7.0%	34.0	50884 (100)	2152 (100)	4.2	13.1
0-9	1416 (1.8)	10 (0.2)	0.7	0.4	1362 (2.7)	11 (0.5)	0.8	0.4
10-19	2475 (3.2)	8 (0.1)	0.3	0.3	2128 (4.2)	6 (0.3)	0.3	0.2
20-29	14306 (18.3)	32 (0.6)	0.2	1.2	8707 (17.1)	19 (0.9)	0.2	0.7
30-39	18052 (23.1)	169 (3.1)	0.9	6.6	11487 (22.6)	49 (2.3)	0.4	2.0
40-49	16258 (20.8)	499 (9.1)	3.1	23.7	10005 (19.7)	140 (6.5)	1.4	6.7
50-59	13274 (17.0)	1107 (20.1)	8.3	67.7	8124 (16.0)	323 (15.0)	4.0	19.7
60-69	7034 (9.0)	1615 (29.3)	23.0	150.4	5023 (9.9)	649 (30.2)	12.9	56.6
70-79	3620 (4.6)	1279 (23.2)	35.3	207.6	2488 (4.9)	558 (25.9)	22.4	85.1
80 -	1769 (2.3)	789 (14.3)	44.6	279.2	1536 (3.0)	397 (18.4)	25.8	108.8

Health, Peru. Among men, reported cases were mostly observed among individuals aged 30-39 years (23.1%), followed by those aged 40-49 years (20.8%), and those aged 20-29 years (18.3%). In contrast, most deaths were reported among those aged 50 years and above, especially among men aged 60-69 (29.3%) followed by those aged 70-79 (23.2%), aged 50-59 years (20.1%), and aged 80 years and above (14.3%). (Table 1, Figure 1A, 1B). Data show a similar pattern for women. The majority of reported cases occur in females aged 20-69 years, and the majority of reported deaths occur among women aged 50 years or more. More specifically, most reported cases occur among women aged 30-39 (22.6%), followed by women aged 40-49 (19.7%), and 50-59 year olds (16.0%). In contrast, most deaths are reported among those aged 60-69 (30.2%), followed by women aged 70-79 (25.9%), and lastly, women aged 80 years and above (18.4%). Regarding CoVID-19 mortality per 100,000 population, seniors (individuals >70 years of age) were the most affected age group; mortality burden per 100,000 is 279.2 among men aged 80 years and above, and 207.6 among men aged 70-79 years. For women of 80 years of age or more mortality is 108.8 and 85.1 for women aged 70-79 years (Table 1, Figure 1F).

The gender proportions of reported cases by age groups are presented in Figure 1C and Figure 1D. The proportion of cases among men is higher than 50% across all age groups (χ^2 test, p-value<0.001). Similarly, the proportion of male deaths is also higher than 50% except for those aged 10-19 years (χ^2 test, p-value<0.001). Cumulative morbidity ratio by gender and age group is presented in Figure 1E, indicating that cumulative morbidity ratio among men is higher than women across all age groups (proportion test, p-value < 0.001) except for individuals aged 0-9 years (proportion test, p-value =0.85). Figure 1F illustrates the mortality per 100,000 population directly caused by CoVID-19 by gender and age group. Mortality is higher than among females aged 20 years and above (proportion test, p-value <0.05), and it is not significantly different among those aged 0-19 years.

Figure 2 shows the cumulative cases and deaths of CoVID-19 by age group for males and females (A through J) over time. The figure suggests cumulative deaths increases after an increase in cumulative cases. The growth curve for overall cumulative cases (all age groups) for men and women appears to increase exponentially until around day 60 (April 29th, 2020), while exponential growth in cumulative deaths overall (all age groups) for men and women appears to occur until around day 70 (May 9th, 2020).

Figure 3 illustrates observed and model based posterior estimates of the crude CFR by age group (A-J) and time-delay adjusted CFR by age group (K-T) for men

and women. Black dots show crude case fatality risks, and light and dark indicate 95% and 50% credible intervals (CrI) for posterior estimates, respectively.

Overall, our model based crude CFR fitted the observed data well, except for individuals aged 80 years and above, probably influenced by low reporting rate/ ascertainment bias of cases at an early stage. Crude CFR for most of age groups increased at the early stage of the epidemic, peaked amidst the outbreak day 34 (April 3rd, 2020) and followed a decreasing trend turning into an almost flat curve.

Overall, our model-based posterior estimates for the time-delay adjusted CFR are substantially higher than the crude observed CFR. These estimates fluctuated at the early stage of the epidemic and then followed a decreasing trend.

The most recent estimates, as of May 25, 2020, of the time-delay adjusted CFR for men and women are 10.8% (95%CrI: 10.5-11.1%) and 6.5% (95%CrI: 6.2-6.8%), respectively, while overall national estimate is 9.1% (95%CrI: 8.9-9.3%) (Figure 4 and Table 2). Among men, senior citizens appear to be severely affected; the

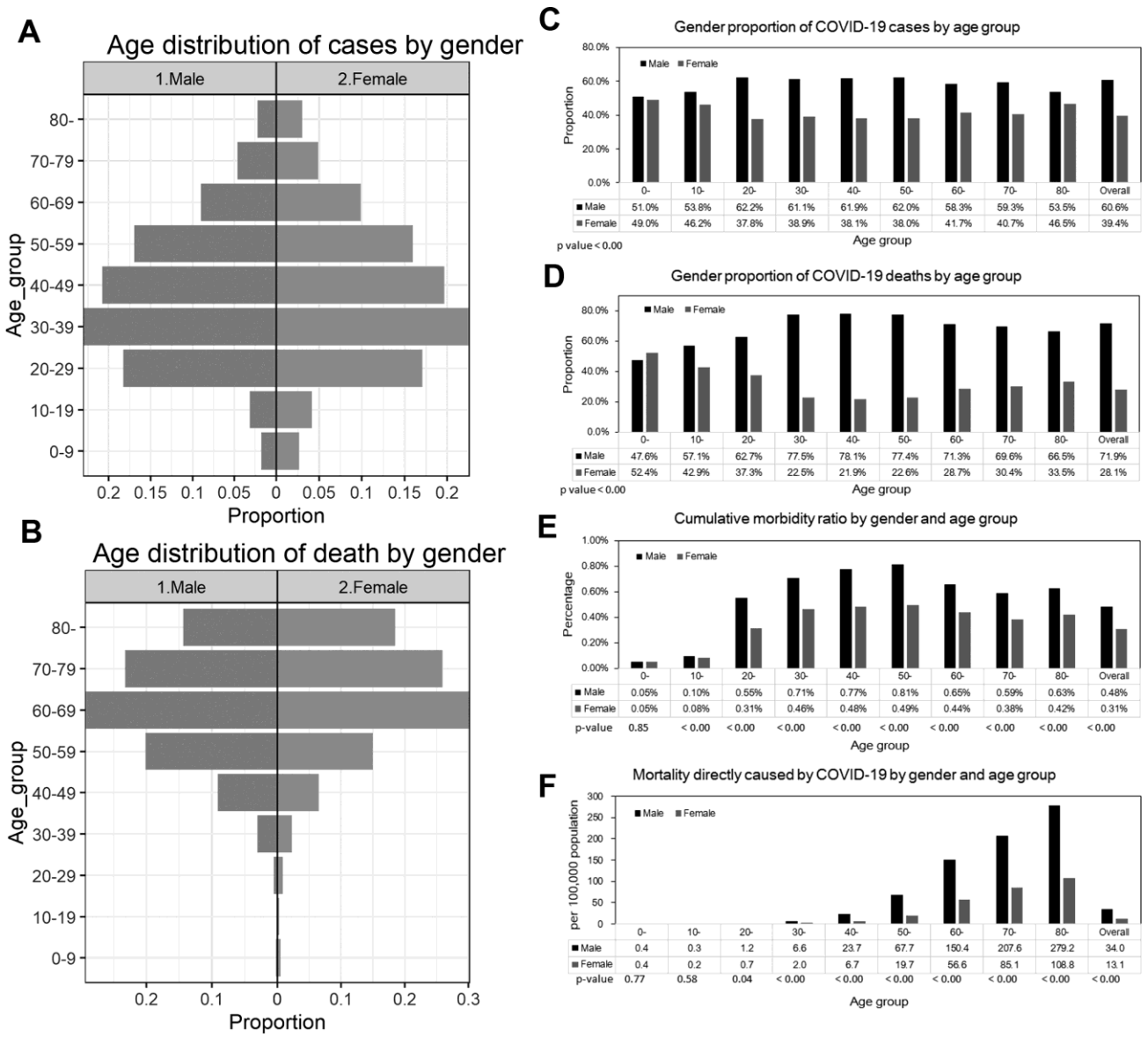

Figure 1. Epidemiological characterization of CoVID-19 in Peru, as of May 25, 2020. (**A**) Age distribution of reported cases by gender, (**B**) Age distribution of reported deaths by gender. (**C**) Gender proportion of CoVID-19 cases by age group, (**D**) Gender proportion of CoVID-19 deaths by age group, (**E**) Cumulative morbidity risk by gender and age group, (**F**) Mortality directly caused by CoVID-19 by gender and age group.

adjusted CFR is 33.1% (95%CrI: 31.7-34.6%) for men aged 60-69 years, 49.4% (95%CrI: 47.3-51.6%) for those aged 70-79 years, and 64.3% (95%CrI: 60.9-67.8%) for those 80 years old and above. We observe a similar pattern for women. The adjusted CFR is 19.2% (95%CrI: 17.9-20.6%) for women aged 60-69 years, 32.2% (95%CrI: 29.9-34.7%) for those aged 70-79 years, and 35.1% (95%CrI: 32.1-38.1%) for women aged 80 years old or more.

DISCUSSION

This study estimates the time-delay adjusted CFR by age group for the ongoing CoVID-19 epidemic in Peru. The crude CFR varies across countries due to differences in testing and timing of tests [29]. The results from our analysis show that the CoVID-19 epidemic in Peru disproportionately impacts senior individuals, especially those who are 70 years of age or older, consistent with CFR estimates obtained from recent studies conducted in China [30, 31], Chile [32], and Italy [33, 34]. This pattern suggests that an aging population could aggravate the fatality impact of CoVID-19, influenza and respiratory syncytial virus [32], as was probably an important factor for its high impact in Italy [33, 34]. While the population in Lain America, including Peru, is aging at a rapid rate, still a relatively small percentage of the population in the region are older than 65 years of age [35]. Hence, the age structure in the region could favor a lower overall CFR than would be expected otherwise with a relatively older population, as in other regions.

Our estimate of adjusted CFR among men (10.8% (95%CrI: 10.5-11.1%)) is 1.7-fold higher than the estimated adjusted CFR for women (6.5% (95%CrI: 6.2-6.8%)), consistent with the estimates given in ref [37]. Men aged 80 years or older have an estimated adjusted CFR as high as 64.3% (95%CrI: 60.9-67.8%), 58-fold higher than our estimates for men aged 0-9, and 1.3-fold higher than our estimates for men aged 70-79. Similarly, the adjusted CFR estimates for women of aged 80 years or older are as high as 35.1% (95%CrI: 32.1-38.1%), 29-fold

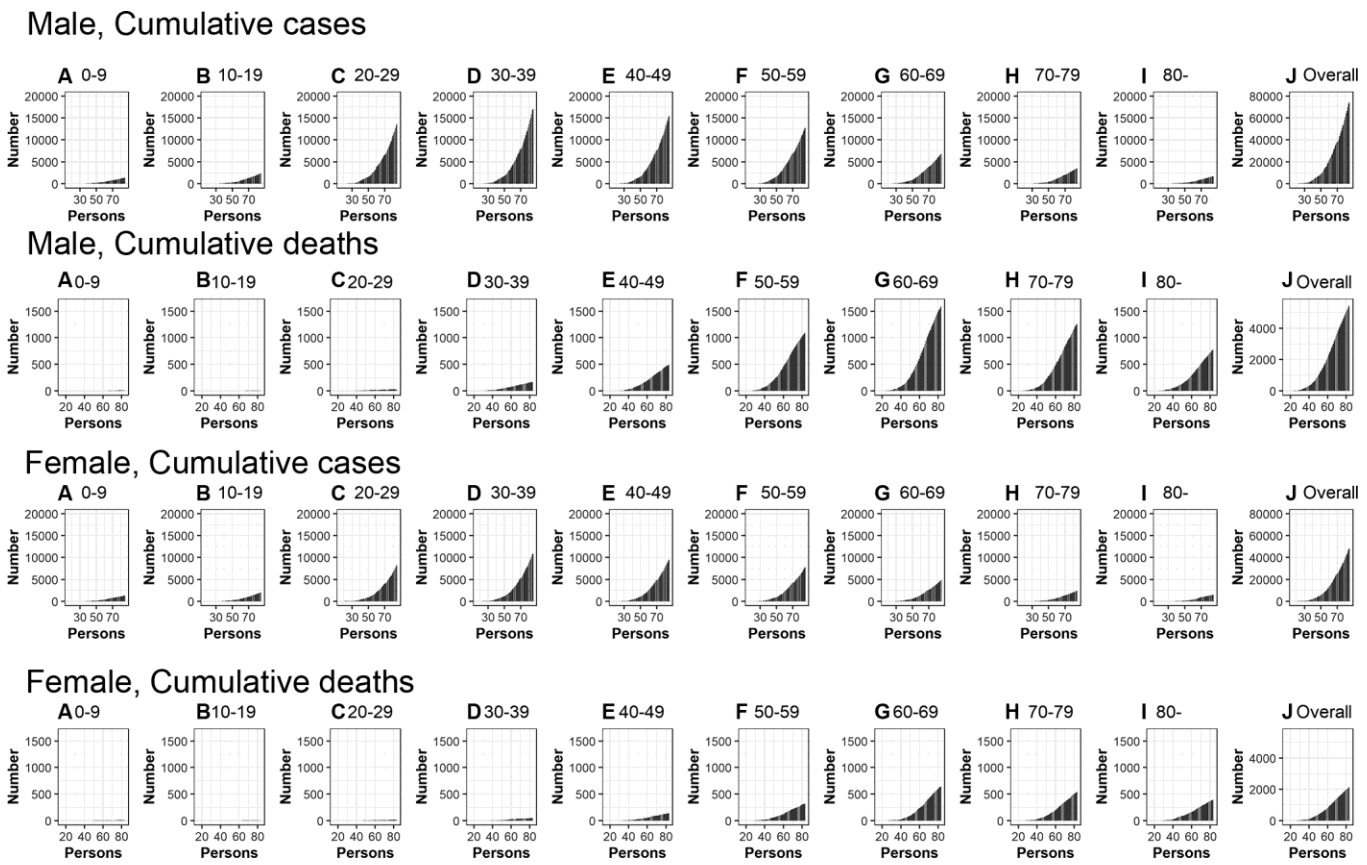

Figure 2. Temporal distribution of cases and deaths by age group due to CoVID-19, March-May 2020, Peru. Top: Male, cumulative cases, Second top: Male, cumulative cases, Second bottom: Female, cumulative cases, Bottom: Female cumulative deaths (**A**) aged 0-9, (**B**) aged 10-19, (**C**) aged 20-29, (**D**) aged 30-39, (**E**) aged 40-49, (**F**) aged 50-59, (**G**) aged 60-69, (**H**) aged 70-79, (**I**) aged 80- and (**J**) Overall (all age groups). Day 1 corresponds to March 1st in 2020.

higher than the estimates obtained for female aged 0-9 and 1.1-fold higher than the estimates obtained for female aged 70-79, consistent with recent findings in Chile [32]. In comparison, a study conducted in China, reported much lower estimates of CFR for individuals >80 years of age (13.4%) [31].

An upward trend in the crude CFR for overall population suggests the disease transmission may be spreading to more vulnerable populations. The majority of social distancing measures in Peru were implemented between March 11-March 18, 2020. However, since 72.4% of the economically active population works in informal jobs, which are concentrated in the poorest areas of the country, compliance with government mitigation strategies can be challenging despite the government's efforts to support the population [37]. Another factor possibly contributing to the upward trend in crude CFR may be an increase in unreported cases due to saturated testing capacity [29]. However, since Peru's testing capacity has substantially increased since the beginning of the outbreak, going from >0.01 test per 1000 population to 0.09 per 1000 in May 22 [15], and the positivity rate estimated at 8.6% for March, 2020, this seems an unlikely cause. In Peru, about 85% of ICU beds with ventilators are currently occupied by patients [37], therefore our present estimates are not affected by excess deaths due to health

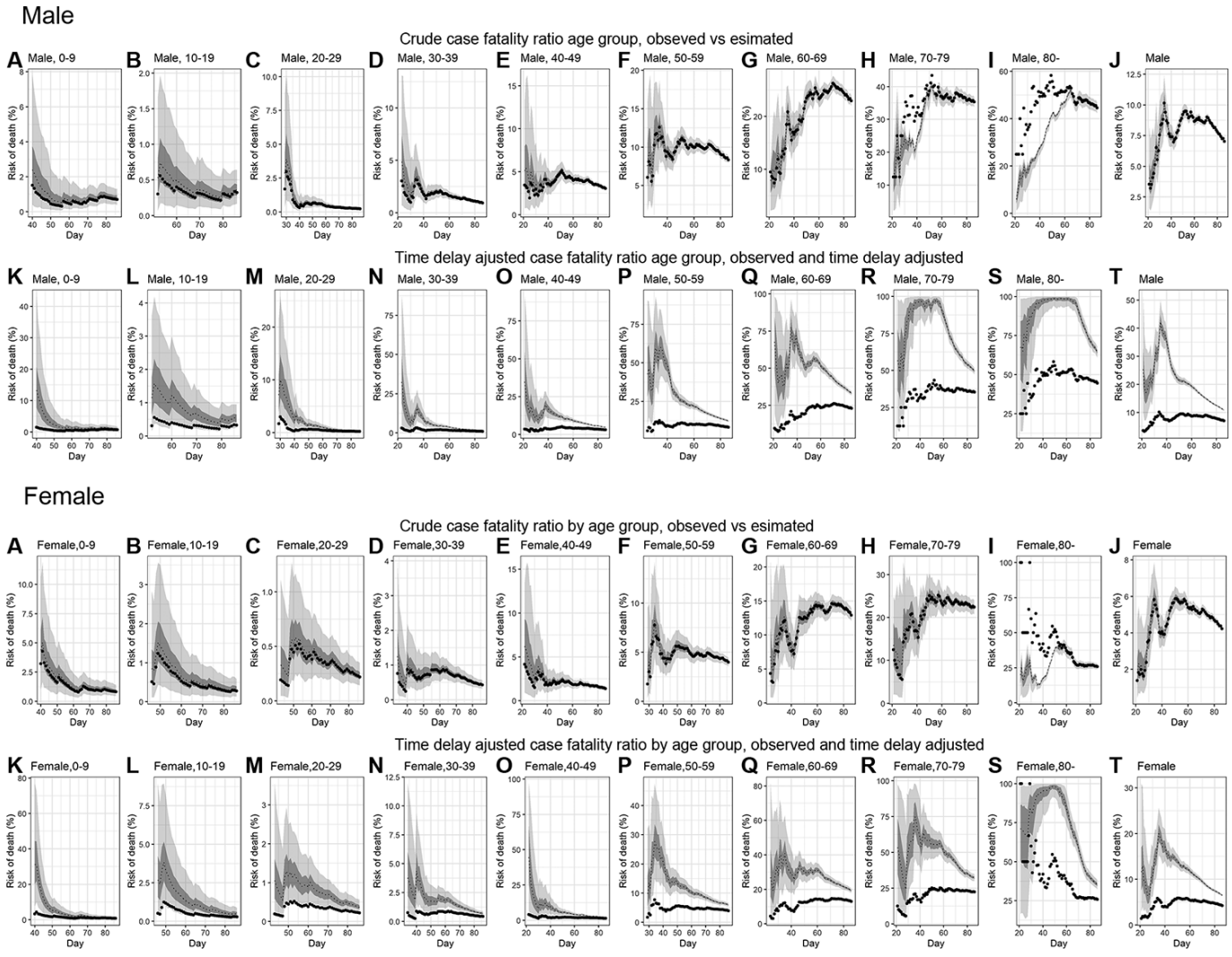

Figure 3. Temporal variation of male and female risk of death by age group caused by CoVID-19, March-May 2020, Peru. Upper two rows; Male risk of deaths, Lower two rows; Female risk of deaths. Observed and posterior estimated of crude case fatality risk of (**A**) aged 0-9, (**B**) aged 10-19, (**C**) aged 20-29, (**D**) aged 30-39, (**E**) aged 40-49, (**F**) aged 50-59, (**G**) aged 60-69, (**H**) aged 70-79, (**I**) aged 80-, (**J**) all age groups and time-delay adjusted case fatality risk of (**K**) aged 0-9, (**L**) aged 10-19, (**M**) aged 20-29, (**N**) aged 30-39, (**O**) aged 40-49, (**P**) aged 50-59, (**Q**) aged 60-69, (**R**) aged 70-79, (**S**) aged 80-, (**T**) all age groups. Day 1 corresponds to March 1st in 2020. Black dots show crude case fatality risk, and light and dark indicates 95% and 50% credible intervals for posterior estimates, respectively.

care demand exceeding health care capacity. However, as the epidemic continues to expand, healthcare capacity may be reached in the short term [37]. Furthermore, the results show an increasing trend in crude CFR around day 45 (May 14th, 2020), probably reflecting the exponential increase of cumulative cases around day 40 (May 9th, 2020).

The downward trend in the adjusted CFR at the early stage may indicate the existence of a reporting delay and the shift of the outbreak to a less vulnerable segment of the population. In particular, the observed differences in estimates between the crude CFR and adjusted CFR can be attributed to the time-delay that is assumed fixed during the course of the epidemic.

The relatively small proportion of males (53.5%) among CoVID-19 cases in the individuals aged 80 years and above can be attributed to the relatively small male population size for that age group; with men comprising only 1.7% of the population >80 years of age in Peru, consistent with estimates for Chile [32]. As higher mortality among male has been reported in China and the U.S. [38], additional data on deaths stratified by gender provides the opportunity to examine the CFR by gender and age.

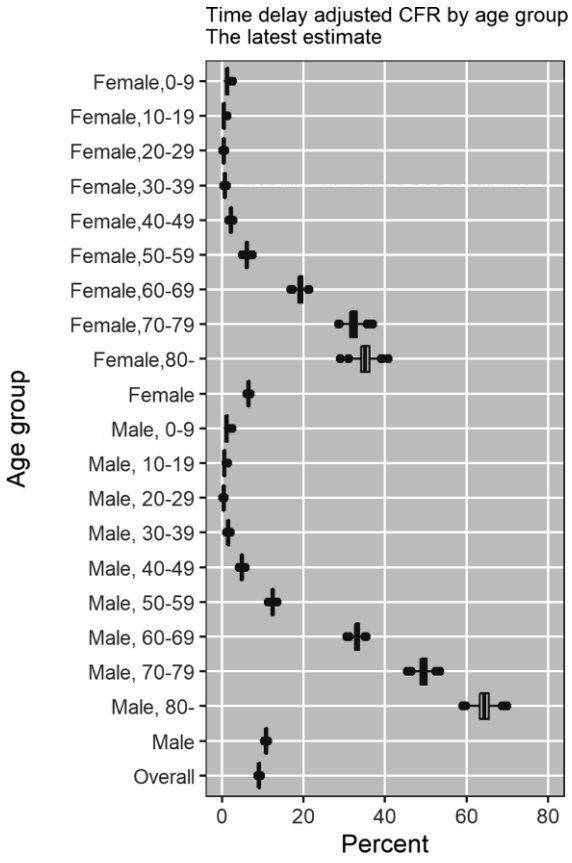

Figure 4. Most recent estimates of time-delay adjusted risk of death caused by CoVID-19 by age group and gender, March-May 2020, Peru. Distribution of time-delay adjusted risk of death from the latest estimates (May 25, 2020) is presented. Top to bottom: female aged 0-9, female aged 10-19, female aged 20-29, female aged 30-39, female aged 40-49, female aged 50-59, female aged 60-69, female aged 70-79, female aged 80 and over, female overall.

Several studies documenting the IFR of CoVID-19 have been reported based on an observational study [39], modeling studies [31, 40] and serological studies [41, 42]. While IFR estimates may be more realistic indicators compared to estimates derived from observed cases alone [43, 44], the external validity of these serological studies, e.g., whether the results can be applied to the generalized population in the region where they are performed, needs to be closely examined, as pointed out elsewhere [40, 45, 46]. In particular, to derive IFR estimates, prevalence, the cumulative number of infected people, is estimated based on the result of serological studies. Then, the cumulative number of deaths in the region is divided by the estimated cumulative number of infected individuals.

Indeed, serological studies based on blood donors and outpatients/hospitalized patients will easily lead to overestimation and underestimation, respectively, because the number of infected individuals is expected to be lower among the blood donors and higher among the outpatients/hospitalized patients. In contrast, the death risk derived from the CFR is less affected by the sampling bias and a convenient indicator to identify the vulnerable subpopulations, especially focusing on a single country with relatively uniform testing capacity across the population.

Our study has at least two limitations. First, our estimates are probably overestimated, due to the effect of under reporting rates and ascertainment rates, as has been underscored in other studies [25, 27, 47]. But a recently enhanced testing capacity in Peru is expected to mitigate these effects, and an ongoing mass serological study will provide data to generate more accurate estimates of the death risk. Second, adjusted CFR, especially among seniors, has displayed fluctuations, highlighting the importance of focusing on sub-group analyses. Additional information such as line lists that include related risks including information on underlying diseases may help to identify subgroups with elevated risks.

CONCLUSIONS

The CoVID-19 pandemic is imposing a large death toll in Peru. Senior individuals, especially those who are

Table 2. Summary results of time-delay adjusted case fatality risk of CoVID-19 in each age group in Peru, 2020 as of May 25, 2020.

Age group	Gender	Latest estimate	Range of median estimates	Crude case fatality rate
Overall		9.1% (95%CrI[a]: 8.9-9.3%)	9.1-32.0%	5.9% (95%CI[b]: 5.8-6.1%) 7660/129148 [c]
Male		10.8% (95%CrI: 10.5-11.1%)	10.8-42.3%	7.0% (95%CI: 6.9-7.2%) 5508/78264
Female		6.5% (95%CrI: 6.2-6.8%)	6.4-20.0%	4.2% (95%CI: 4.1-4.4%) 2152/50884
0-9	Male	1.1% (95%CrI: 0.5-1.8%)	1.0-13.3%	0.7% (95%CI:0.3-1.3%) 10/1416
	Female	1.2% (95%CrI: 0.7-2.0%)	1.2-31.4%	0.8% (95%CI: 0.4-1.4%) 11/1362
10-19	Male	0.5% (95%CrI: 0.3-1.0%)	0.4-1.6%	0.3% (95%CI: 0.1-0.6%) 8/2475
	Female	0.5% (95%CrI: 0.2-0.9%)	0.4-3.8%	0.3% (95%CI: 0.1-0.6%) 6/2128
20-29	Male	0.4% (95%CrI: 0.2-0.5%)	0.4-10.0%	0.2% (95%CI: 0.2-0.3%) 32/14306
	Female	0.4% (95%CrI: 0.2-0.6%)	0.4-1.3%	0.2% (95%CI: 0.1-0.3%) 19/8707
30-39	Male	1.5% (95%CrI: 1.3-1.7%)	1.5-32.3%	0.9% (95%CI: 0.8-1.1%) 169/18052
	Female	0.7% (95%CrI: 0.5-0.9%)	0.9-4.4%	0.4% (95%CI: 0.3-0.6%) 49/11487
40-49	Male	4.8% (95%CrI: 4.4-5.2%)	4.8-34.7%	3.1% (95%CI: 2.8-3.3%) 499/16258
	Female	2.2% (95%CrI: 1.8-2.5%)	2.2-44.6%	1.4% (95%CI: 1.2-1.6%) 140/10005
50-59	Male	12.4% (95%CrI: 11.7-13.1%)	12.4-60.0%	8.3% (95%CI: 7.9-8.8%) 1107/13274
	Female	6.0% (95%CrI: 5.4-6.7%)	7.5-27.7%	4.0% (95%CI: 3.6-4.4%) 323/8124
60-69	Male	33.1% (95%CrI: 31.7-34.6%)	33.1-77.8%	23.0% (95%CI: 22.0-24.0%) 1615/7034
	Female	19.2% (95%CrI: 17.9-20.6%)	19.2-40.9%	12.9% (95%CI: 12.0-13.9%) 649/5023
70-79	Male	49.4% (95%CrI: 47.3-51.6%)	48.7-97.8%	35.3% (95%CI: 33.8-36.9%) 1279/3620
	Female	32.2% (95%CrI: 29.9-34.7%)	24.1-74.9%	22.4% (95%CI: 20.8-24.1%) 558/2488
80-	Male	64.3% (95%CrI: 60.9-67.8%)	64.3-98.9%	44.6% (95%CI: 42.3-47.0%) 789/1769
	Female	35.1% (95%CrI: 32.1-38.1%)	35.1-98.3%	25.8% (95%CI: 23.7-28.1%) 397/1536

[a] 95%CrI: 95% credibility intervals (CrI), [b] 95%CI: 95% confidence interval, [c] Cumulative cases over cumulative deaths

older than 70 years of age, are being disproportionately affected by the CoVID-19 pandemic, particularly elderly men. CFR was as high as 64.3% (95%CrI: 60.9-67.8%) for men aged 80 older, 58-fold higher than our estimates for men aged 0-9. The overall adjusted CFR in Peru is estimated to be higher than in other countries, which is worrying, particularly because healthcare demand has not yet exceeded capacity, but probably will do in the coming weeks. The relatively younger age structure in Latin America may help ameliorate the overall CFR than would otherwise be expected with an older age structure in the population.

MATERIALS AND METHODS

Data

We obtained daily cumulative numbers of reported laboratory confirmed CoVID-19 cases and deaths stratified by age group and gender through May 25, 2020. Different age groups had different starting times, which correspond to the day when death was reported. Confirmed CoVID-19 cases were retrieved from three surveillance systems: a) national surveillance system (confirmed and suspected cases based on a case definition), b) Netlab system (molecular test) and c) SICOVID system (rapid serological test). CoVID-19 deaths were obtained from two surveillance systems: a) national surveillance system (confirmed and suspected deaths based on a case definition) and b) Vital statistics system (National System of mortality -SINADEF- which is an online system that keeps track of death certificates) [48]. A suspected case presents with acute respiratory infection and with two or more of the following symptoms (cough, sore throat, respiratory distress, nasal congestion or fever), close contact with a CoVID-19 case within 14 days of symptoms onset, or people who live or traveled to cities with community transmission of SARS-CoV-2 within 14 days of symptoms onset. On the other hand, the definition of confirmed cases is a suspected case with a positive lab test. [49].

Population size by age, group, and gender in 2020 were retrieved from the Ministry of Health in Peru [50].

Statistical analysis

The crude CFR is defined as the number of cumulative deaths over the number of cumulative cases. For the estimation of CFR in real time, we employed the delay from hospitalization to death, h_s, which is assumed to be given by $h_s = H(s) - H(s-1)$ for $s>0$ where $H(s)$ is a cumulative density function of the delay from hospitalization to death and follows a gamma distribution with mean 10.1 days and SD 5.4 days, as given in ref, Mizumoto and Chowell [24]. Let $\pi_{a,ti}$ be the time-delay adjusted case fatality risk on reported day t_i in area a, the likelihood function of the estimate π_{a,t_i} is given by equation:

$$L(\pi_{a,t_i}; c_{a,t}, D_{a,t}) = \prod_{t_i} \left(\sum_{t=1}^{t_i} c_{a,t} \atop D_{a,t_i} \right) \left(\pi_{a,t_i} \frac{\sum_{t=2}^{t_i} \sum_{s=1}^{t-1} c_{a,t-s} h_s}{\sum_{t=1}^{t_i} c_{a,t}} \right)^{D_{a,t_i}} \left(1 - \pi_{a,t_i} \frac{\sum_{t=2}^{t_i} \sum_{s=1}^{t-1} c_{a,t-s} h_s}{\sum_{t=1}^{t_i} c_{a,t}} \right)^{\sum_{t=1}^{ti} c_{a,t} - D_{a,t_i}}$$

where $c_{a,t}$ represents the number of new cases with reported day t in area a, and D_{a,t_i} is the cumulative number of deaths until reported day t_i in area a [51, 52]. Among the cumulative cases with reported day t in area a, D_{a,t_i} have died and the remainder have survived the infection. The contribution of those who have died with biased death risk is shown in the middle parenthetical term and the contribution of survivors is presented in the right parenthetical term. We assume that D_{a,t_i} is the result of the binomial sampling process with probability π_{a,t_i}.

We used a Monte Carlo Markov Chain (MCMC) method in a Bayesian framework to estimate model parameters. We evaluated the convergence of MCMC chains using the potential scale reduction statistic [53, 54]. Estimates and 95% credibility intervals for these estimates are based on the posterior probability distribution of each parameter and samples drawn from the posterior distributions. All statistical analyses were conducted in R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) using the 'rstan' package.

AUTHOR CONTRIBUTIONS

GC and KM conceived the early study idea. KM implemented statistical analysis. AT and KM wrote the first full draft. CM performed data acquisition. All authors contributed to the revision of the manuscript. AU advised the study, and revised the manuscript. GC advised on and helped shape the research. All authors contributed to the interpretation of the results and edited and commented on several earlier versions of the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors report no conflicts of interest.

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REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. Lancet Infect Dis. 2020; 20:533–34. https://doi.org/10.1016/S1473-3099(20)30120-1 PMID:32087114

2. Heymann DL, Shindo N, and WHO Scientific and Technical Advisory Group for Infectious Hazards. COVID-19: what is next for public health? Lancet. 2020; 395:542–45. https://doi.org/10.1016/S0140-6736(20)30374-3 PMID:32061313

3. World Bank. (2020). The Economy in the Time of CoVID-19. In: Bank W, ed. Semiannual Report of the Latin America and Caribbean Region. (Washington DC: World Bank).

4. Lloyd-Sherlock P, Ebrahim S, Geffen L, McKee M. Bearing the brunt of covid-19: older people in low and middle income countries. BMJ. 2020; 368:m1052. https://doi.org/10.1136/bmj.m1052 PMID:32169830

5. Burki T. COVID-19 in latin america. Lancet Infect Dis. 2020; 20:547–48. https://doi.org/10.1016/S1473-3099(20)30303-0 PMID:32311323

6. Bedford J, Enria D, Giesecke J, Heymann DL, Ihekweazu C, Kobinger G, Lane HC, Memish Z, Oh MD, Sall AA, Schuchat A, Ungchusak K, Wieler LH, and WHO Strategic and Technical Advisory Group for Infectious Hazards. COVID-19: towards controlling of a pandemic. Lancet. 2020; 395:1015–18. https://doi.org/10.1016/S0140-6736(20)30673-5 PMID:32197103

7. Rodriguez-Morales AJ, Gallego V, Escalera-Antezana JP, Méndez CA, Zambrano LI, Franco-Paredes C, Suárez JA, Rodriguez-Enciso HD, Balbin-Ramon GJ, Savio-Larriera E, Risquez A, Cimerman S. COVID-19 in latin america: the implications of the first confirmed case in Brazil. Travel Med Infect Dis. 2020; 35:101613. https://doi.org/10.1016/j.tmaid.2020.101613 PMID:32126292

8. The Lancet. COVID-19: learning from experience. Lancet. 2020; 395:1011. https://doi.org/10.1016/S0140-6736(20)30686-3 PMID:32222181

9. Navarro JC, Arrivillaga-Henríquez J, Salazar-Loor J, Rodriguez-Morales AJ. COVID-19 and dengue, co-epidemics in Ecuador and other countries in latin america: pushing strained health care systems over the edge. Travel Med Infect Dis. 2020; 101656. https://doi.org/10.1016/j.tmaid.2020.101656 PMID:32268196

10. Lorenz C, Azevedo TS, Chiaravalloti-Neto F. COVID-19 and dengue fever: a dangerous combination for the health system in Brazil. Travel Med Infect Dis. 2020; 35:101659. https://doi.org/10.1016/j.tmaid.2020.101659 PMID:32278756

11. MOH. (2020). Sala Situacional COVID-19 Peru.

12. Aquino M and Garrison C. (2020). Peru records first confirmed case of coronavirus, President Vizcarra says. Rueters.

13. Explorer A. (2020). Coronavirus in Peru- the latest updates. (Amazon Explorer).

14. Munayco CV, Tariq A, Rothenberg R, Soto-Cabezas GG, Reyes MF, Valle A, Rojas-Mezarina L, Cabezas C, Loayza M, Chowell G, and Peru COVID-19 working group. Early transmission dynamics of COVID-19 in a southern hemisphere setting: lima-peru: February 29th-March 30th, 2020. Infect Dis Model. 2020; 5:338–45. https://doi.org/10.1016/j.idm.2020.05.001 PMID:32399507

15. Hasell J, Ortiz-Ospina E, Mathieu E, Ritchie H, Roser M. (2020). To understand the global pandemic, we need global testing – the Our World in Data COVID-19 Testing dataset. Our World in data.

16. Rainisch G, Undurraga EA, Chowell G. A dynamic modeling tool for estimating healthcare demand from the COVID19 epidemic and evaluating population-wide interventions. Int J Infect Dis. 2020; 96:376–83. https://doi.org/10.1016/j.ijid.2020.05.043 PMID:32425631

17. Cascella M, Rajnik M, Cuomo A, Dulebohn SC, Di Napoli R. Features, Evaluation and Treatment Coronavirus (COVID-19). 2020. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020. PMID:32150360

18. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19.

Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

19. Wu JT, Leung K, Bushman M, Kishore N, Niehus R, de Salazar PM, Cowling BJ, Lipsitch M, Leung GM. Estimating clinical severity of COVID-19 from the transmission dynamics in Wuhan, China. Nat Med. 2020; 26:506–10. https://doi.org/10.1038/s41591-020-0822-7 PMID:32284616

20. Stokes EK, Zambrano LD, Anderson KN, Marder EP, Raz KM, El Burai Felix S, Tie Y, Fullerton KE. Coronavirus disease 2019 case surveillance — United States, January 22-May 30, 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:759–65. https://doi.org/10.15585/mmwr.mm6924e2 PMID:32555134

21. CDC COVID-19 Response Team. Severe outcomes among patients with coronavirus disease 2019 (COVID-19) - United States, February 12-March 16, 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:343–46. https://doi.org/10.15585/mmwr.mm6912e2 PMID:32214079

22. Epidemiology Working Group for NCIP Epidemic Response, Chinese Center for Disease Control and Prevention. [The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19) in China]. Zhonghua Liu Xing Bing Xue Za Zhi. 2020; 41:145–151. https://doi.org/10.3760/cma.j.issn.0254-6450.2020.02.003 PMID:32064853

23. Reed C, Biggerstaff M, Finelli L, Koonin LM, Beauvais D, Uzicanin A, Plummer A, Bresee J, Redd SC, Jernigan DB. Novel framework for assessing epidemiologic effects of influenza epidemics and pandemics. Emerg Infect Dis. 2013; 19:85–91. https://doi.org/10.3201/eid1901.120124 PMID:23260039

24. Mizumoto K, Chowell G. Estimating risk for death from coronavirus disease, China, January-February 2020. Emerg Infect Dis. 2020; 26:1251–56. https://doi.org/10.3201/eid2606.200233 PMID:32168464

25. Li R, Pei S, Chen B, Song Y, Zhang T, Yang W, Shaman J. Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV-2). Science. 2020; 368:489–93. https://doi.org/10.1126/science.abb3221 PMID:32179701

26. Jewell NP, Lei X, Ghani AC, Donnelly CA, Leung GM, Ho LM, Cowling BJ, Hedley AJ. Non-parametric estimation of the case fatality ratio with competing risks data: an application to severe acute respiratory syndrome (SARS). Stat Med. 2007; 26:1982–98. https://doi.org/10.1002/sim.2691 PMID:16981181

27. Lipsitch M, Donnelly CA, Fraser C, Blake IM, Cori A, Dorigatti I, Ferguson NM, Garske T, Mills HL, Riley S, Van Kerkhove MD, Hernán MA. Potential biases in estimating absolute and relative case-fatality risks during outbreaks. PLoS Negl Trop Dis. 2015; 9:e0003846. https://doi.org/10.1371/journal.pntd.0003846 PMID:26181387

28. Pan A, Liu L, Wang C, Guo H, Hao X, Wang Q, Huang J, He N, Yu H, Lin X, Wei S, Wu T. Association of public health interventions with the epidemiology of the COVID-19 outbreak in Wuhan, China. JAMA. 2020; 323:1–9. https://doi.org/10.1001/jama.2020.6130 PMID:32275295

29. Asahi K, Undurraga EA, Wagner R. Benchmarking the CoVID-19 pandemic across countries and states in the U.S.A. under heterogeneous testing. medRxiv. 2020. [Epub ahead of print]. https://doi.org/10.1101/2020.05.01.20087882

30. Deng X, Yang J, Wang W, Wang X, Zhou J, Chen Z, Li J, Chen Y, Yan H, Zhang J, Zhang Y, Wang Y, Qiu Q, et al. Case fatality risk of the first pandemic wave of novel coronavirus disease 2019 (COVID-19) in China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa578 PMID:32409826

31. Verity R, Okell LC, Dorigatti I, Winskill P, Whittaker C, Imai N, Cuomo-Dannenburg G, Thompson H, Walker PG, Fu H, Dighe A, Griffin JT, Baguelin M, et al. Estimates of the severity of coronavirus disease 2019: a model-based analysis. Lancet Infect Dis. 2020; 20:669–77. https://doi.org/10.1016/S1473-3099(20)30243-7 PMID:32240634

32. Undurraga EA, Chowell G, Mizumoto K. Case fatality risk by age from COVID-19 in a high testing setting in Latin America: Chile, March-May, 2020. medRxiv. 2020. [Epub ahead of print]. https://doi.org/10.1101/2020.05.25.20112904

33. Onder G, Rezza G, Brusaferro S. Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.4683 PMID:32203977

34. Dowd JB, Andriano L, Brazel DM, Rotondi V, Block P, Ding X, Liu Y, Mills MC. Demographic science aids in understanding the spread and fatality rates of COVID-19. Proc Natl Acad Sci USA. 2020; 117:9696–98.

https://doi.org/10.1073/pnas.2004911117
PMID:32300018

35. Ham-Chande R, Nava-Bolaños I. Convergence Toward Demographic Aging in Latin America and the Caribbean. Annu Rev Sociol. 2019; 45:607–623. https://doi.org/10.1146/annurev-soc-073018-022532

36. Martínez AD. (2020). Peru Passes Coronavirus Risk to the Working Class (disponible en español). (nacla).

37. Castillo M. (2020). Peru seemed to do everything right. So how did it become a Covid-19 hotspot? CNN.

38. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, et al, and the Northwell COVID-19 Research Consortium. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the new york city area. JAMA. 2020; 323:2052–59. https://doi.org/10.1001/jama.2020.6775 PMID:32320003

39. Russell TW, Hellewell J, Jarvis CI, van Zandvoort K, Abbott S, Ratnayake R, Cmmid Covid-Working Group, Flasche S, Eggo RM, Edmunds WJ, Kucharski AJ. Estimating the infection and case fatality ratio for coronavirus disease (COVID-19) using age-adjusted data from the outbreak on the diamond princess cruise ship, February 2020. Euro Surveill. 2020; 25:2000256. https://doi.org/10.2807/1560-7917.ES.2020.25.12.2000256 PMID:32234121

40. Mizumoto K, Kagaya K, Chowell G. Early epidemiological assessment of the transmission potential and virulence of coronavirus disease 2019 (COVID-19) in Wuhan City: China, January-February, 2020. medRxiv. 2020. https://doi.org/10.1101/2020.02.12.20022434

41. Ioannidis J. The infection fatality rate of COVID-19 inferred from seroprevalence data. medRxiv. 2020. [Epub ahead of print]. https://doi.org/10.1101/2020.05.13.20101253

42. To KKW, Cheng VCC, Cai JP, Chan KH, Chen LL, Wong LH, et al. Lancet Microbe. https://doi.org/10.1016/S2666-5247(20)30053-7

43. Wong JY, Wu P, Nishiura H, Goldstein E, Lau EH, Yang L, Chuang SK, Tsang T, Peiris JS, Wu JT, Cowling BJ. Infection fatality risk of the pandemic a(H1N1)2009 virus in hong kong. Am J Epidemiol. 2013; 177:834–40. https://doi.org/10.1093/aje/kws314 PMID:23459950

44. Presanis AM, De Angelis D, Hagy A, Reed C, Riley S, Cooper BS, Finelli L, Biedrzycki P, Lipsitch M, and New York City Swine Flu Investigation Team. The severity of pandemic H1N1 influenza in the United States, from April to July 2009: a bayesian analysis. PLoS Med. 2009; 6:e1000207. https://doi.org/10.1371/journal.pmed.1000207 PMID:19997612

45. Wu X, Fu B, Chen L, Feng Y. Serological tests facilitate identification of asymptomatic SARS-CoV-2 infection in Wuhan, China. J Med Virol. 2020; 10:1002. https://doi.org/10.1002/jmv.25904 PMID:32311142

46. Giugliano F. Mass Coronavirus Antibody Tests Have Serious Limits. Bloomberg Opinion. April 24, 2020.

47. Ghani AC, Donnelly CA, Cox DR, Griffin JT, Fraser C, Lam TH, Ho LM, Chan WS, Anderson RM, Hedley AJ, Leung GM. Methods for estimating the case fatality ratio for a novel, emerging infectious disease. Am J Epidemiol. 2005; 162:479–86. https://doi.org/10.1093/aje/kwi230 PMID:16076827

48. Ministerio de Salud, Peru: Alerta Epidemiológica ante la transmisión de COVID-IS en el Perú. https://www.dge.gob.pe/portal/docs/alertas/2020/AE016.pdf.

49. Vargas-Herrera J, Ruiz KP, Nuñez GG, Ohno JM, Pérez-Lu JE, Huarcaya WV, Clapham B, Cortez-Escalante J. [Preliminary results of the strengthening of the national death registry information system]. Rev Peru Med Exp Salud Publica. 2018; 35:505–14. https://doi.org/10.17843/rpmesp.2018.353.3913 PMID:30517488

50. Instituto Nacional de Estadística e Informáticas. PERÚ: Estimaciones y Proyecciones de Población por Departamento, Sexo y Grupos Quinquenales de Edad 1995-2025. Boletín de Análisis Demográfico No 37. Lima, Setiembre 2009. https://www.inei.gob.pe/media/principales_indicadores/libro_1.pdf

51. Nishiura H, Klinkenberg D, Roberts M, Heesterbeek JA. Early epidemiological assessment of the virulence of emerging infectious diseases: a case study of an influenza pandemic. PLoS One. 2009; 4:e6852. https://doi.org/10.1371/journal.pone.0006852 PMID:19718434

52. Tsuzuki S, Lee H, Miura F, Chan YH, Jung SM, Akhmetzhanov AR, Nishiura H. Dynamics of the pneumonic plague epidemic in Madagascar, August to October 2017. Euro Surveill. 2017; 22:17–00710. https://doi.org/10.2807/1560-7917.ES.2017.22.46.17-00710 PMID:29162211

53. Gamerman D, Lopes HF. (2006). Markov chain Monte Carlo: stochastic simulation for Bayesian inference: CRC Press).

54. Gelman A, Rubin DB. Inference from iterative simulation using multiple sequences. Stat Sci. 1992; 7:457–472. https://doi.org/10.1214/ss/1177011136

Research Paper

Development and validation of a risk stratification model for screening suspected cases of COVID-19 in China

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ABSTRACT

How to quickly identify high-risk populations is critical to epidemic control. We developed and validated a risk prediction model for screening SARS-CoV-2 infection in suspected cases with an epidemiological history. A total of 1019 patients, ≥13 years of age, who had an epidemiological history were enrolled from fever clinics between January 2020 and February 2020. Among 103 (10.11%) cases of COVID-19 were confirmed. Multivariable analysis summarized four features associated with increased risk of SARS-CoV-2 infection, summarized in the mnemonic COVID-19-REAL: radiological evidence of pneumonia (1 point), eosinophils < 0.005 × 10^9/L (1 point), age ≥ 32 years (2 points), and leukocytes < 6.05 × 10^9 /L (1 point). The area under the ROC curve for the training group was 0.863 (95% CI, 0.813 - 0.912). A cut-off value of less than 3 points for COVID-19-REAL was assigned to define the low-risk population. Only 10 (2.70%) of 371 patients were proved to be SARS-CoV-2 positive, with a negative predictive value of 0.973. External validation was similar. This study provides a simple, practical, and robust screening model, COVID-19-REAL, able to identify populations at high risk for SARS-CoV-2 infection.

INTRODUCTION

At the end of December 2019, an outbreak of pneumonia caused by a novel coronavirus (severe acute respiratory syndrome coronavirus 2, SARS-CoV-2) was reported in Wuhan, China [1]. Transmission takes place through respiratory droplets and other routes such as ocular surfaces [2–4]. This highly contagious virus spread rapidly to other cities of China, and gave rise to a global outbreak. As of Mar 23, 2020, over 300,000 cases of COVID-19 have been confirmed worldwide, and more than 10,000 have died. The number of confirmed cases is still increasing. One study estimates the basic reproductive number (R0) to be 2.68, and the epidemic doubling time to be 6.4 days [5]. The control of COVID-19 must include detection and isolation of latent infection. A considerable proportion of COVID-19 cases are infected by those who only had mild

symptoms [6, 7]. COVID-19 patients have the highest viral load near symptom presentation [8]. Moreover, the rapid spread of COVID-19 has meant that large numbers of patients with suspicious symptoms are often crowded into fever clinics for diagnosis.

At present, cases are confirmed by a positive result with high-throughput sequencing or real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) assay of samples from nasal or pharyngeal swabs [9]. However, nucleic acid tests are not available to all suspected patients in pandemic areas due to the shortage of equipment and reagents [10, 11]. Testing for all cases with mild symptoms and/or an epidemiological history can lead to competition for resources. In addition, undiagnosed mild-type COVID-19 patients who were not properly isolated could become sources of infection as their viral load peaks near symptom presentation, which could explain the rapid spread of this epidemic [12]. A large proportion of infected cases continue to test negative for viral RNA, even after they develop clinical manifestations, and positive chest CT (computed tomography) results [13, 14]. This dilemma demands a fast and accurate model for early screening for SARS-CoV-2 infections to prioritize high-risk patients for clinical care, isolation, and contact tracking. Previous studies reported that a number of COVID-19 patients exhibit lymphopenia and thrombocytopenia [15–17]. Blood counts and high-sensitivity C-reactive protein (hsCRP) are commonly used for early identification of fever [18], and CT is used to assess pneumonia. These tests are simple and fast, and nearly all patients with fever or respiratory symptoms can be tested. We first compared alterations of hematological parameters between cases with and without SARS-CoV-2 infection, then developed and validated a novel score-based prognostic model (COVID-19-REAL) for SARS-CoV-2 infection.

RESULTS

Patient characteristics

A total of 1019 patients were enrolled in this study out of the 1076 patients who presented to fever clinics until 5 February 2020. Fifty-seven patients were excluded, including one with stroke, two with organ transplantation, one with HIV, 12 with cancer, one with active tuberculosis, 18 with age < 12 years, and 22 unconfirmed cases until 10 February 2020 (Figure 1). Of the 1019 patients, 485 (48%) were female, and the median age was 34 years (range 13 to 91 years). The characteristics of the patients are shown in Table 1. All received sequencing or nucleic acid testing using RT-PCR; 103 (10.11%) tested positive for SAR-CoV-2 (Supplementary Table 1).

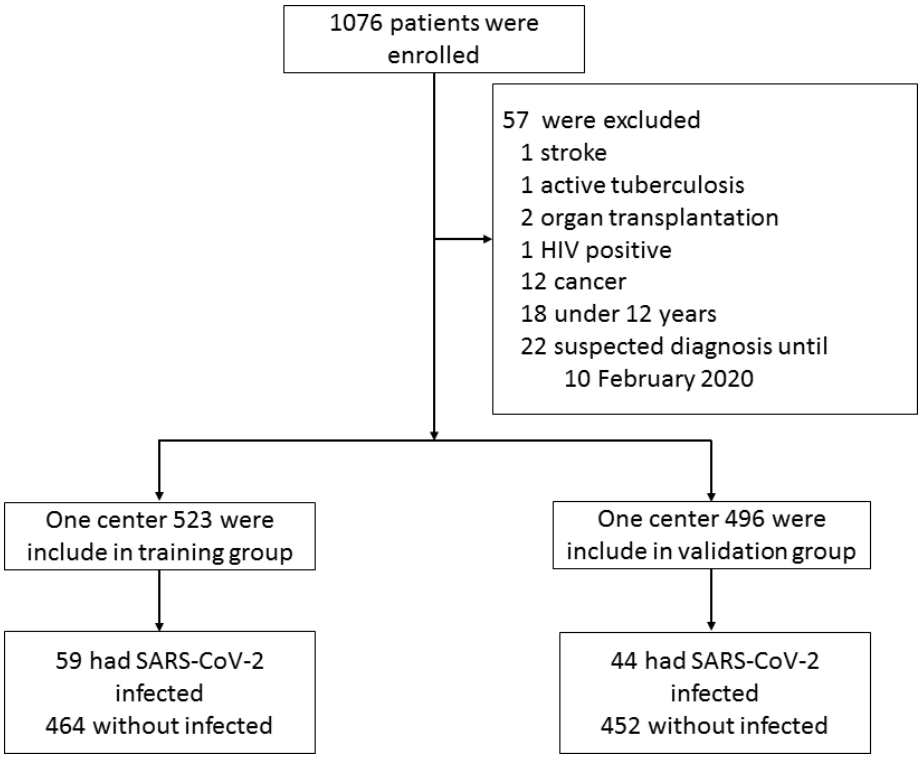

Figure 1. Flowchart of patient selection.

Table 1. Characteristics of patients in this study.

Characteristic	Development group	Validation group	P-value
Number	523	496	
Female	253 (48.38%)	232 (46.77%)	0.609
Age (years)	33 (24-45)	32 (26-40)	0.895
Symptom			
Fever	412 (78.78%)	367 (73.99%)	0.072
Dry cough	209 (39.96%)	171 (34.48%)	0.070
Fatigue	45 (8.60%)	43 (8.669%)	0.970
Pharyngalgia	84 (16.06%)	89 (17.94%)	0.424
Diarrhea	12 (2.29%)	13 (2.62%)	0.736
Coexisting comorbidity			
Hypertension	29 (5.54%)	34 (6.85%)	0.386
Cardiovascular diseases	6 (1.15%)	5 (1.01%)	0.83
Diabetes	11 (2.10%)	7 (1.41%)	0.48
Chronic lung disease	0 (0.00%)	3 (0.60%)	0.115
Chronic liver disease	11 (2.10%)	19 (3.83%)	0.103
Chronic renal disease	1 (0.19%)	2 (0.40%)	0.615
Blood parameters			
Leucocyte (109/L)	6.9 (5.30-8.80)	7.0 (5.20-9.03)	0.74
hsCRP (mg/L)	5.07 (0.90-15.95)	9.10 (2.75-22.56)	<0.001
Monocyte (109/L)	0.50 (0.40-0.70)	0.55 (0.41-0.76)	0.477
RBC (1012/L)	4.78 (4.44-5.22)	4.74 (4.37-5.14)	0.031
Hematocrit (%)	0.42 (0.40-0.46)	0.42 (0.39-0.46)	0.538
Lymphocyte (109/L)	1.30 (0.90-1.80)	1.25 (0.86-1.69)	0.592
MCH (pg)	30.30 (29.30-31.00)	30.30 (29.48-31.20)	0.074
MCHC (g/L)	339.00 (333.00-345.00)	339.00 (332.00-345.00)	0.251
MPV	10.00 (9.60-10.60)	10.00 (9.40-10.60)	0.04
Basophilic granulocyte (109/L)	0.02 (0.01-0.02)	0.02 (0.01-0.03)	<0.001
Eosinophil (109/L)	0.04 (0.01-0.08)	0.03 (0.01-0.09)	0.612
Hemoglobin (g/L)	143 (133-157)	144.00 (132-156)	0.318
PDW (%)	11.70 (10.80-12.85)	11.20 (10.10-12.60)	0.003
Platelet (109/L)	216 (181-256)	212 (173-256)	0.874
Platelet hematocrit (%)	0.22 (0.18-0.25)	0.21 (0.18-0.25)	0.37
Neutrophil (109/L)	4.70 (3.40-6.60)	4.75 (3.30-7.10)	0.7
Radiological evidence of pneumonia	92 (17.59%)	63 (12.70%)	0.03
Confirmed with COVID-19	59 (11.28%)	44 (8.87%)	0.202

Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; HsCRP: high-sensitivity C-reactive proteins; RBC: red blood cell; MCH: mean corpuscular hemoglobin; MPV: mean platelet volume; MCHC: mean corpuscular hemoglobin concentration; PDW: platelet distribution width; CT: chest computed tomography scan.

Association factors for SARS-CoV-2 infection

The association between age and infection rate is presented in Figure 2A. The rate of SARS-CoV-2 infection increased with age. After stratifying patients by age quartile, the positive rate of SARS-CoV-2 infection from first to fourth quartile was 2.90%, 3.06%, 12.14%, and 23.81% in the training group, and 2.97%, 3.45%, 6.72%, and 23.28% in the validation group (Figure 2B, C). The risk of infection in last two quartiles was relatively higher than the first two quartiles. The infection rate was lower (less than 5%) for patients with age < 32 years. Subgroup analyses were performed for patients with age ≥ 32 years to stratify those as high-risk population.

The factors associated with a positive result of SARS-CoV-2 infection in univariate analysis are shown in

Table 2. Compared to non-COVID-19 patients, COVID-19 patients had a lower count of leukocytes (5.10×10^9/L vs 7.15×10^9/L, $p < 0.001$), monocytes (0.40×10^9/L vs 0.55×10^9/L, $p < 0.001$), lymphocytes (1.10×10^9/L vs 1.30×10^9/L, $p = 0.02$), eosinophils (0.01×10^9/L vs 0.04×10^9/L, $p < 0.001$), neutrophils (3.40×10^9/L vs 5.00×10^9/L, $p < 0.001$), and platelets (192×10^9/L vs 220×10^9/L, $p < 0.001$). They had a higher age (47 years vs 32 years, $p < 0.001$) in the training group, and similar characteristics were found in validation group (Supplementary Tables 2). After multivariate analysis, age, leukocytes, and eosinophils remained as significant factors; lymphocytes, leukocytes, monocytes, platelets, and neutrophils were not significant indicators (Table 2).

A COVID-19 prediction model based on age, leukocyte, and eosinophil and radiological evidence of pneumonia

The AUROC value for the prediction of leukocytes and eosinophils in the training group for COVID-19 diagnosis were 0.747 and 0.729, respectively. This was comparable to the validation group, where the AUROC value for leukocytes and eosinophils were 0.763 and 0.772 (Supplementary Figure 1). Using Youden's index, the optimal cut-off value for leukocytes and eosinophils were 6.05×10^9/L and 0.005×10^9/L.

Significantly higher infection rate was observed in those with leukocytes < 6.05×10^9/L (23.66% vs 4.45% in leukocytes ≥ 6.05×10^9/L), and eosinophils < 0.005×10^9/L (33.72% vs 6.68% in eosinophils ≥ 0.005×10^9/L) in the training group. The trend was consistent in the validation group, where the infection rate was 18.13% vs 3.5% for leukocyte subgroups, and 28.13% vs 4.25% for eosinophil subgroups (Figure 3).

Based on multivariate logistic regression analysis, the major criterion was age ≥ 32 years (2 point). Minor criteria included leukocytes < 6.05×10^9/L (1 point), eosinophils < 0.005×10^9/L (1 point), and radiological evidence of pneumonia (1 point) (Table 3). The model showed good discrimination (AUROC = 0.863, 95% CI, 0.81 - 0.91) and calibration. Internal verification shows AUROC = 0.863 (95% CI, 0.81 - 0.91) and external verification showed good discrimination (AUROC = 0.871, 95% CI, 0.82-0.93) (Table 4, Supplementary Figure 2)

The following four risk groups were developed: very low risk (0 point), with a risk of infection of 0.84%; low risk (1 - 2 points), with a risk of 3.57%; moderate risk (3 points), with a risk of 19.05%; and high risk (4 - 5 points), with a risk of 61.70%. For the validation group, the infection risk was 0% (0 point); 3.49% (1 - 2 points); 10.87% (3 points); and 55.32% (4 - 5 points) (Figure 4). A cut-off value of less than 3 points for COVID-19-REAL was used to stratify 371 out of 523 (70.94%) cases as low risk, of whom only 10 (2.70%) were infected with SARS-CoV-2 in the training group. The remaining 152 patients were classified as higher risk of infection; about 49 (32.24%) were infected with SARS-CoV-2. According to the cut-off value of 3 points, the sensitivity, specificity, positive predictive value, and negative predictive value was 0.778, 0.831, 0.322, and 0.973 respectively (Table 4).

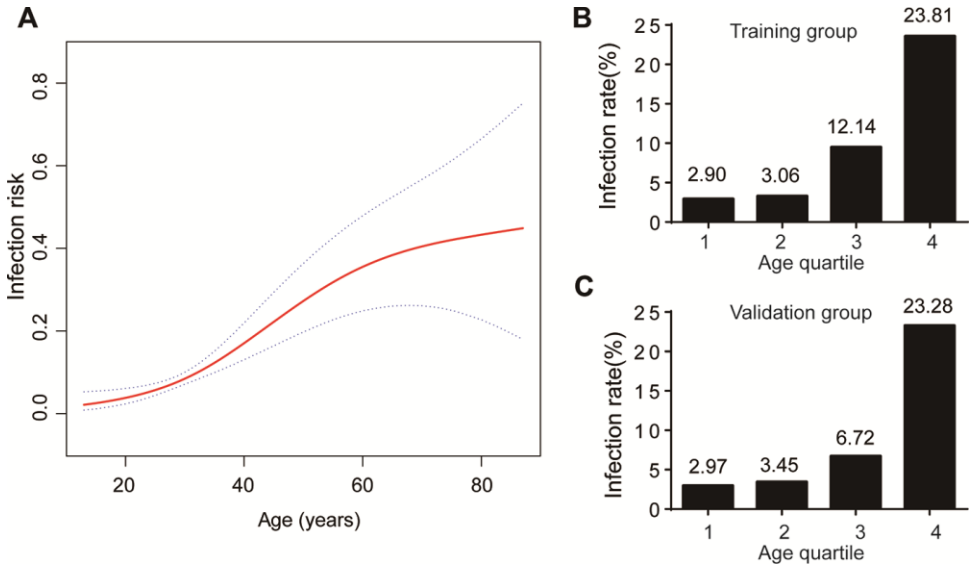

Figure 2. Age and COVID-19 infection. (**A**) The infection risk increased with increasing age; (**B**) Infection rate at age quartile in training group; (**C**) Infection rate at age quartile in validation group.

Table 2. Univariate and multivariate analyses of indicators for SARS-CoV-2 infection in training group.

Variable	non-COVID-19	COVID-19	Univariate		Multivariate	
	N = 464	N = 59	OR (95% CI)	P-value	OR (95% CI)	P-value
Age (years)	32 (23-42)	47 (38-56)	1.05 (1.04- 1.07)	<0.001	1.06 (1.04- 1.08)	<0.001
Leucocyte (109/L)	7.15 (5.70-9.03)	5.10 (4.05-6.05)	0.72 (0.63- 0.83)	<0.001	0.74 (0.64- 0.85)	<0.001
Monocyte (109/L)	0.55 (0.40-0.70)	0.40 (0.30-0.50)	0.06 (0.01- 0.24)	<0.001		
RBC (1012/L)	4.80 (4.45-5.24)	4.70 (4.25-5.01)	0.46 (0.27- 0.78)	0.004		
Lymphocyte (109/L)	1.30 (0.90-1.90)	1.10 (0.85-1.50)	0.57 (0.35- 0.91)	0.019		
Basophilic granulocyte (109/L)	0.02 (0.01-0.03)	0.01 (0.01-0.02)	0.00 (0.00- 45.46)	0.098		
Eosinophil (107/L)	4.00 (1.00-9.00)	1.00 (0.00-3.00)	0.88 (0.82- 0.95)	0.001	0.91 (0.85- 0.98)	0.009
Platelet (109/L)	220.00 (184.00-259.00)	192.00 (144.50-234.00)	0.99 (0.99- 1.00)	<0.001		
Neutrophil (109/L)	5.00 (3.60-6.80)	3.40 (0.80-22.20)	0.75 (0.65- 0.87)	<0.001		
Radiological evidence of pneumonia	68 (14.66%)	24 (40.68%)	3.99 (2.24- 7.13)	<0.001	4.00 (2.04- 7.86)	<0.001

Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; RBC: red blood cell; HsCRP: high-sensitivity C-reactive proteins; CT: chest computed tomography scan; CI: confidence interval; OR: odds ratio.

DISCUSSION

Beginning in mid-January 2020, a large number of people living in Wuhan left the area via public transportation due to Chinese New Year, leading to a dramatic increase in confirmed or suspected cases nationwide. The management of these suspected cases is of major concern. Nucleic acid testing is currently the main diagnostic method, but the sensitivity and specificity of nucleic acid tests are yet to be verified, and the overall detection rate is constrained by virus concentration and sampling method. Another problem is that some patients with positive chest CT images test negative for COVID-19 by RT-PCR [14]. With such issues in mind, we proposed a robust, high-throughput screening model to help prioritize high-risk patients. We used the data of routine blood tests and CT images to develop a score system (COVID-19-REAL) that can stratify patients into risk groups. Suspected cases with 0 - < 3 points had a predicted probability of 99.16% in training and 97.3% in validation groups for not being infected by SARS-CoV-2. This risk classification can be employed by clinicians and medical institutions, especially those with inadequate detection reagents or equipment, to make rational allocation of resources.

Previous investigations have revealed valuable information about demographics for COVID-19. Most patients with COVID-19 are older [16]. We first stratified patients according to age. Two earlier studies stated the median age of the patients was 56 and 59 years [15, 19]. In our study, the median age was 47 years. We found the risk of infection significantly increased with age, from less than 3% to over 23% from the first to last quartile.

The level of leukocytes, monocytes, lymphocytes, eosinophils, neutrophils, and platelets was dramatically lower in COVID-19 patients. Our results are consistent with previous research that patients exhibited leukopenia, lymphopenia, and thrombocytopenia after SARS-CoV-2 infection [15, 20]. Some researchers suggested a decreased level of white blood cells could serve as an auxiliary diagnosis [20]. Similar patterns emerged in SARS-CoV, with cases of lymphopenia and neutropenia [21, 22], and decreased levels of leukocytes and platelets [23]. A SARS-CoV model showed that neutrophils, lymphocytes, and leukocytes were significantly reduced the day after infection [24]. In a SARS-CoV MA15 infection model, the decrease of peripheral blood cells was explained by inflammatory cell infiltration to the lungs [25]. The N protein of

SARS-CoV enhances eosinophilic infiltration into the lungs and aggravates lung inflammation [26]. Lung lesions were the most important feature of SARS-CoV-2 infections [20], and eosinophilopenia may indicate a poor prognosis of COVID-19 [27]. These results shed light on the neglected role that eosinophils might play in the progression of respiratory disease.

To better stratify SARS-CoV-2 infection risk for the suspected cases, four criteria including leukocytes < 6.05×10^9 /L (1 point), eosinophils < 0.005×10^9/L (1 point), radiological evidence of pneumonia (1 point), and age ≥ 32 years (2 point) were used to determine the likelihood of SARS-CoV-2 infection. We defined four risk groups: very low risk (0 point), low risk (1 - 2 points), moderate risk (3 points), and high risk (4 - 5 points). According to the cut-off value that was assigned as less than 3 points of COVID-19-REAL score, the number of suspected cases who required priority examination and hospitalization decreased by 70.94% and 71.98%, while maintaining a false negative rate of 2.70% and 2.24% in training and validation group, respectively.

Clinical decision models have been explored to predict infection of SARS-CoV-2. Sun et al. [28] studied 788 cases in Singapore to identify populations at high risk for COVID-19. From their large population-based study, a model that combined laboratory blood tests, clinical findings, and radiology was proposed, and the AUROC was 0.88 (95% CI: 0.83- 0.93). Similar to our cohort, those authors found that eosinophils and CT imaged pneumonia were strong predictors. However, their conclusions were limited by a lack of external verification, clinical inapplicability caused by redundant parameters, and missing data in laboratory blood tests.

The advantage of present study is that a simple and applicable prediction model, COVID-19-REAL, which combines age, radiological image, and two functionally related hematological indicators (i.e., leukocytes and eosinophils) has been developed to stratify and distinguish between high- and low-risk populations suspected of SARS-CoV-2 infection. This evaluation of suspected cases based on age, radiological image, and two dichotomous criteria could be easily implemented in routine clinical practice. In clinical settings where resources and testing kits are limited, patients with advanced respiratory symptoms are usually tested first. However, those undiagnosed mild-type COVID-19 patients who were not properly isolated would become sources of infection as the viral load peaked near symptom presentation. This score system will be of great help for early infection screening and offer more information for physicians to help prioritize high-risk patients.

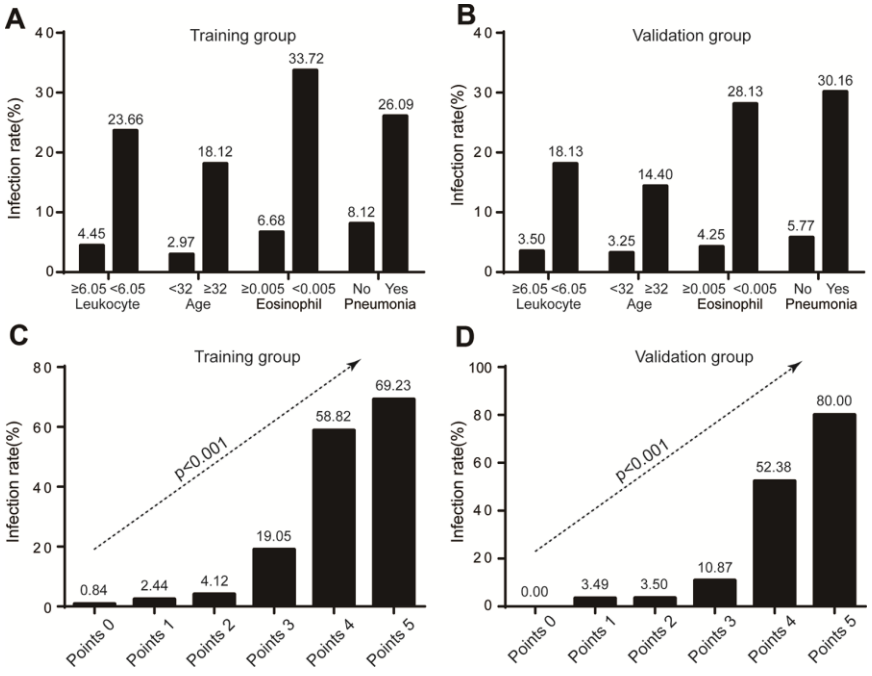

Figure 3. Infection rate in risk stratification. (**A**) Infection rate stratified by leukocyte, age, eosinophil, and radiological evidence of pneumonia in training group; (**B**) Infection rate stratified by leukocyte, age, eosinophil, and radiological evidence of pneumonia in validation group; (**C**) Infection rate according to COVID-19-REAL score in training group; (**D**) Infection rate according to COVID-19-REAL score in validation group.

Table 3. Multivariate analyses of indicators for SARS-CoV-2 infection in training group.

Variable	OR (95% CI)	β Coefficient (95% CI)	P-value	Point score
Age (years)				
<32 (n = 236)	1	1		
≥32(n = 287)	8.63 (3.60 - 20.64)	2.16 (1.28- 3.03)	<0.001	2
Eosinophil (109/L)				
>0.005 (n = 437)	1	1		
≤0.005 (n = 86)	4.92 (2.50 - 9.69)	1.59 (0.94 - 2.27)	<0.001	1
Leucocyte (109/L)				
>6.05 (n =337)	1	1		
≤6.05 (n =186)	6.23 (3.14 - 12.35)	1.83 (1.14 - 2.51)	<0.001	1
Radiological evidence				
No Pneumonia(n = 431)	1	1		
Pneumonia(n = 92)	3.73 (1.83 - 7.62)	1.32 (0.60 – 2.03)	<0.001	1

Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; CT: chest computed tomography scan; CI: confidence interval; OR: odds ratio.

Table 4. Performances of the risk stratification algorithm in the diagnosis of SARS-CoV-2 infection in training and validation groups.

Group	AUROC (95% CI)	Specificity	Sensitivity	Positive PV	Negative PV
Training group	0.863 (0.813-0.912)	0.778	0.831	0.322	0.973
Validation group	0.871 (0.816-0.925)	0.772	0.818	0.259	0.978

Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; CI: confidence interval; Positive PV: positive predictive value; Negative PV: negative predictive value.

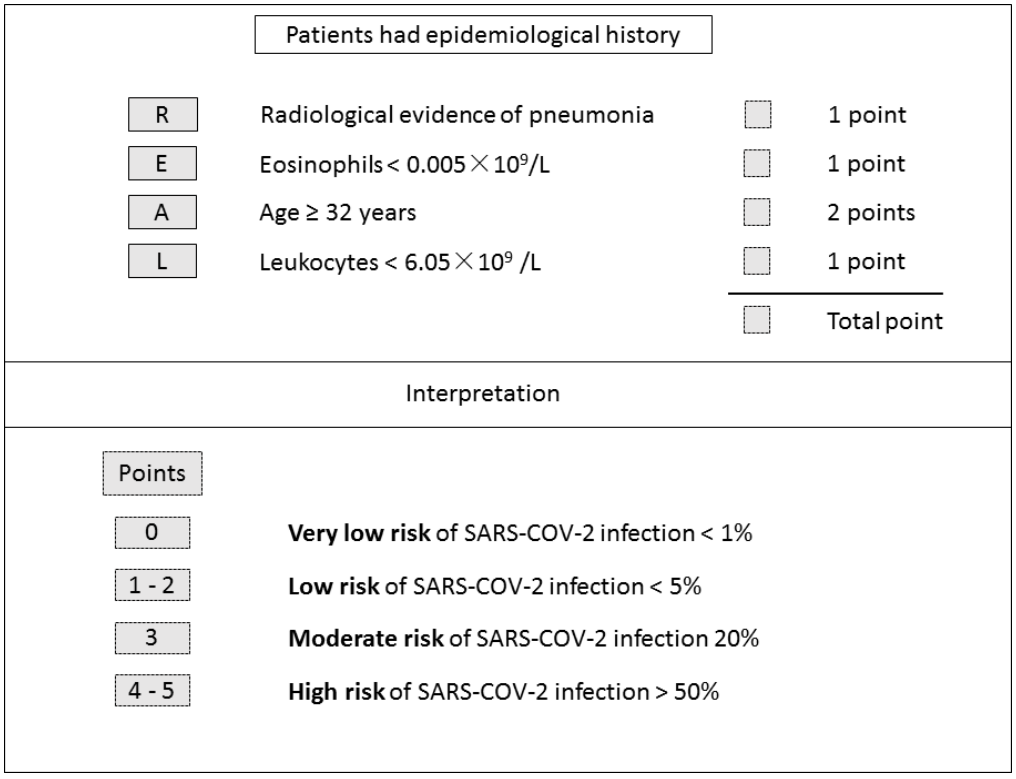

Figure 4. COVID-19-REAL model for risk stratification of SARS-CoV-2 infection.

There are limitations in current study. Our training and validation data comes from China; their applicability to Western populations must be separately evaluated. The results were obtained from people over 12 years of age, and may not be applicable to younger people. Only routine tests including hsCRP, radiological image, and blood cell count were performed, and other hematological indicators including liver and kidney function are lacking.

In conclusion, this study provides a simple, practical, and robust screening model (COVID-19-REAL) to identify high risk populations for SARS-CoV-2 infection. This prediction model will help reduce the burden on hospitals in pandemic areas and help them allocate resources more rationally.

MATERIALS AND METHODS

Patients

Suspect cases of COVID-19 with age ≥13 years with an epidemiological history were included from fever clinics of the First Affiliated Hospital, College of Medicine, Zhejiang University and Taizhou Enze Medical Center (Group), Enze Hospital, between 23 January 2020 and 5 February 2020. All suspected cases received sequencing or RT-PCR assay for SARS-CoV-2. According to National Health Commission, an epidemiological history of COVID-19 is defined as follows: within 14 days before the onset of the disease (1) there were tourism or residence histories of Wuhan or its surrounding areas, or other communities with confirmed cases; (2) there were contacts with confirmed cases of COVID-19; (3) there were contacts with suspected cases (having fever or respiratory symptoms) from Wuhan or its surrounding areas, or other communities with confirmed cases; (4) one confirmed case was found in an enclosed environment (such as a family house, a construction site, an office, etc.), with one or more cases of fever/respiratory tract infection re found at the same time

The patient-selection process is shown in Figure 1. The COVID-19 cases were all confirmed by sequencing or RT-PCR assay [9]. The RT-PCR was mainly performed using a commercial kit for SARS-CoV-2 detection (BoJie, Shanghai, China) which was approved by China Food and Drug Administration. We excluded patients with HIV infection, cancer, organ transplantation, stoke, active tuberculosis, severe and critical COVID-19 patients according to the National Health Commission [17], and suspected cases without confirmed laboratory evidence until 10 February 2020. The study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, and complied with the ethical guidelines of the Declaration of Helsinki. The researchers only analyzed anonymous data, so informed consent was waived. Age, gender, laboratory assessments consisting of hsCRP, complete blood count, and radiological images were obtained from electronic medical records. Radiological evidence of pneumonia was defined as lung consolidation and/or ground-glass opacity [20]. The images were reviewed independently by two radiologists, and if there were disagreements, a third radiologist would perform further examination.

Statistical analysis

Continuous variables were expressed as medians and interquartile range (IQR), and were compared by t-test or Mann–Whitney U-test. Chi-squared test or Fisher's exact test was used to compare categorical variables and expressed as percentages. Generalized linear models with a logit link were used to test the association between age and the risk of COVID-19 infection. Univariate and multivariate analyses were performed to identify indicators of COVID-19 patients. Variables with $P < 0.1$ in a univariate analysis were then included in a forward stepwise regression model. A score for the final model was developed by rounding the coefficients of the logit model. Predicted and observed risk was calculated for each score. The area under receiver operating characteristic (AUROC) curve was used to assess the accuracy of different scores in diagnosis power. Internal validation was performed using a bootstrap procedure with 500 bootstrapped samples. The Youden's index was used to determine the optimal cut-off level for predicting clinical outcomes. All statistical analysis was performed by Statistical Package for the Social Sciences version 19.0 (International Business Machines Corporation, Armonk, NY) and R version 3.4 (R Foundation, Vienna, Austria). All tests were two tailed and $P < 0.05$ was considered to indicate statistical significance.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and

China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

2. Cabrini L, Landoni G, Zangrillo A. Minimise nosocomial spread of 2019-nCoV when treating acute respiratory failure. Lancet. 2020; 395:685. https://doi.org/10.1016/S0140-6736(20)30359-7 PMID:32059800

3. Patel A, Jernigan DB, and 2019-nCoV CDC Response Team. Initial public health response and interim clinical guidance for the 2019 novel coronavirus outbreak — United States, December 31, 2019-February 4, 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:140–46. https://doi.org/10.15585/mmwr.mm6905e1 PMID:32027631

4. Lu CW, Liu XF, Jia ZF. 2019-nCoV transmission through the ocular surface must not be ignored. Lancet. 2020; 395:e39. https://doi.org/10.1016/S0140-6736(20)30313-5 PMID:32035510

5. Wu JT, Leung K, Leung GM. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. Lancet. 2020; 395:689–97. https://doi.org/10.1016/S0140-6736(20)30260-9 PMID:32014114

6. Qiu J. Covert coronavirus infections could be seeding new outbreaks. Nature. 2020. [Epub ahead of print]. https://doi.org/10.1038/d41586-020-00822-x PMID:32203376

7. Li R, Pei S, Chen B, Song Y, Zhang T, Yang W, Shaman J. Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV-2). Science. 2020; 368:489–93. https://doi.org/10.1126/science.abb3221 PMID:32179701

8. To KK, Tsang OT, Leung WS, Tam AR, Wu TC, Lung DC, Yip CC, Cai JP, Chan JM, Chik TS, Lau DP, Choi CY, Chen LL, et al. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect Dis. 2020; 20:565–74. https://doi.org/10.1016/S1473-3099(20)30196-1 PMID:32213337

9. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

10. Pang J, Wang MX, Ang IY, Tan SH, Lewis RF, Chen JI, Gutierrez RA, Gwee SX, Chua PE, Yang Q, Ng XY, Yap RK, Tan HY, et al. Potential rapid diagnostics, vaccine and therapeutics for 2019 novel coronavirus (2019-nCoV): a systematic review. J Clin Med. 2020; 9:623. https://doi.org/10.3390/jcm9030623 PMID:32110875

11. Onder G, Rezza G, Brusaferro S. Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.4683 PMID:32203977

12. Zheng S, Fan J, Yu F, Feng B, Lou B, Zou Q, Xie G, Lin S, Wang R, Yang X, Chen W, Wang Q, Zhang D, et al. Viral load dynamics and disease severity in patients infected with SARS-CoV-2 in Zhejiang province, China, January-March 2020: retrospective cohort study. BMJ. 2020; 369:m1443. https://doi.org/10.1136/bmj.m1443 PMID:32317267

13. Xiao SY, Wu Y, Liu H. Evolving status of the 2019 novel coronavirus infection: Proposal of conventional serologic assays for disease diagnosis and infection monitoring. J Med Virol. 2020; 92:464–467. https://doi.org/10.1002/jmv.25702 PMID:32031264

14. Xie X, Zhong Z, Zhao W, Zheng C, Wang F, Liu J. Chest CT for typical 2019-nCoV pneumonia: relationship to negative RT-PCR testing. Radiology. 2020; 296:E41-45. https://doi.org/10.1148/radiol.2020200343 PMID:32049601

15. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

16. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

17. Wu J, Liu J, Zhao X, Liu C, Wang W, Wang D, Xu W, Zhang C, Yu J, Jiang B, Cao H, Li L. Clinical characteristics of imported cases of COVID-19 in jiangsu province: a multicenter descriptive study. Clin Infect Dis. 2020. [Epub ahead of print].

https://doi.org/10.1093/cid/ciaa199
PMID:32109279

18. Yo CH, Hsieh PS, Lee SH, Wu JY, Chang SS, Tasi KC, Lee CC. Comparison of the test characteristics of procalcitonin to c-reactive protein and leukocytosis for the detection of serious bacterial infections in children presenting with fever without source: a systematic review and meta-analysis. Ann Emerg Med. 2012; 60:591–600.
https://doi.org/10.1016/j.annemergmed.2012.05.027
PMID:22921165

19. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, Ren R, Leung KS, Lau EH, Wong JY, Xing X, Xiang N, Wu Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med. 2020; 382:1199–207.
https://doi.org/10.1056/NEJMoa2001316
PMID:31995857

20. Song F, Shi N, Shan F, Zhang Z, Shen J, Lu H, Ling Y, Jiang Y, Shi Y. Emerging 2019 novel coronavirus (2019-nCoV) pneumonia. Radiology. 2020; 295:210–17.
https://doi.org/10.1148/radiol.2020200274
PMID:32027573

21. Liu J, Zheng X, Tong Q, Li W, Wang B, Sutter K, Trilling M, Lu M, Dittmer U, Yang D. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. J Med Virol. 2020; 92:491–94.
https://doi.org/10.1002/jmv.25709
PMID:32056249

22. Li YH, Hu CY, Wu NP, Yao HP, Li LJ. Molecular characteristics, functions, and related pathogenicity of MERS-CoV proteins. Engineering (Beijing). 2019; 5:940–47.
https://doi.org/10.1016/j.eng.2018.11.035
PMID:32288963

23. Ahmed AE, Al-Jahdali H, Alshukairi AN, Alaqeel M, Siddiq SS, Alsaab H, Sakr EA, Alyahya HA, Alandonisi MM, Subedar AT, Aloudah NM, Baharoon S, Alsalamah MA, et al. Early identification of pneumonia patients at increased risk of middle east respiratory syndrome coronavirus infection in Saudi Arabia. Int J Infect Dis. 2018; 70:51–56.
https://doi.org/10.1016/j.ijid.2018.03.005
PMID:29550445

24. Chu YK, Ali GD, Jia F, Li Q, Kelvin D, Couch RC, Harrod KS, Hutt JA, Cameron C, Weiss SR, Jonsson CB. The SARS-CoV ferret model in an infection-challenge study. Virology. 2008; 374:151–63.
https://doi.org/10.1016/j.virol.2007.12.032
PMID:18234270

25. Gralinski LE, Sheahan TP, Morrison TE, Menachery VD, Jensen K, Leist SR, Whitmore A, Heise MT, Baric RS. Complement activation contributes to severe acute respiratory syndrome coronavirus pathogenesis. mBio. 2018; 9:e01753–18.
https://doi.org/10.1128/mBio.01753-18
PMID:30301856

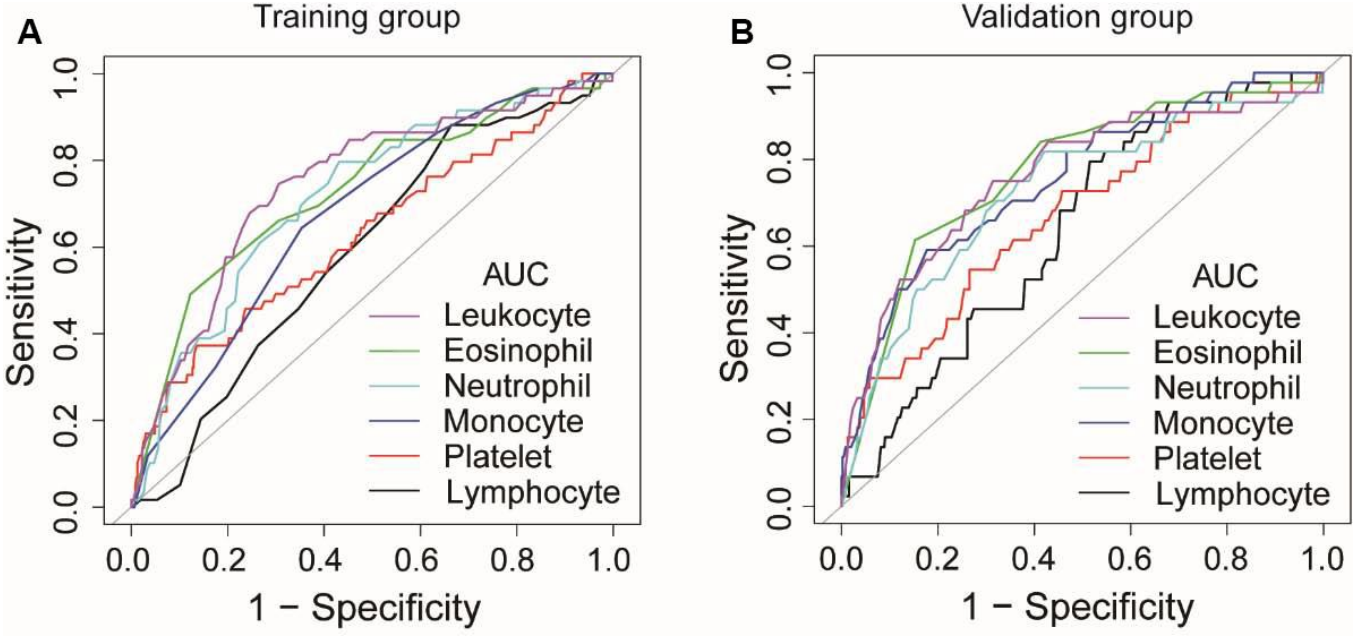
26. Yasui F, Kai C, Kitabatake M, Inoue S, Yoneda M, Yokochi S, Kase R, Sekiguchi S, Morita K, Hishima T, Suzuki H, Karamatsu K, Yasutomi Y, et al. Prior immunization with severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) nucleocapsid protein causes severe pneumonia in mice infected with SARS-CoV. J Immunol. 2008; 181:6337–48.
https://doi.org/10.4049/jimmunol.181.9.6337
PMID:18941225

27. Du Y, Tu L, Zhu P, Mu M, Wang R, Yang P, Wang X, Hu C, Ping R, Hu P, Li T, Cao F, Chang C, et al. Clinical features of 85 fatal cases of COVID-19 from Wuhan. A retrospective observational study. Am J Respir Crit Care Med. 2020; 201:1372–79.
https://doi.org/10.1164/rccm.202003-0543OC
PMID:32242738

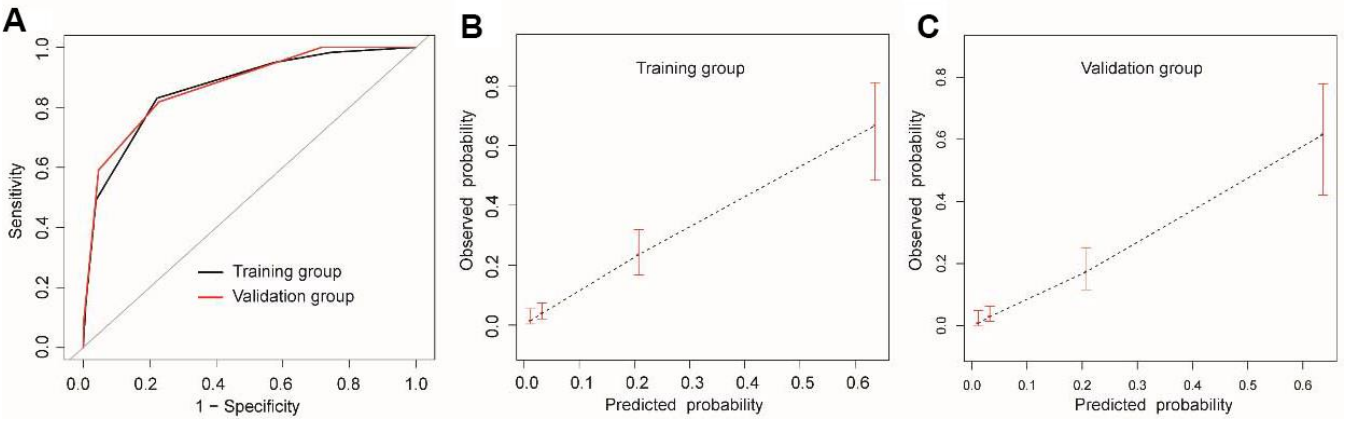
28. Sun Y, Koh V, Marimuthu K, Ng OT, Young B, Vasoo S, Chan M, Lee VJ, De PP, Barkham T, Lin RT, Cook AR, Leo YS. Epidemiological and clinical predictors of COVID-19. Clin Infect Dis. 2020. [Epub ahead of print].
https://doi.org/10.1093/cid/ciaa322
PMID:32211755

SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. AUROC of leukocyte, monocyte, lymphocyte, eosinophil, neutrophil and platelets in COVID-19 diagnosis. (**A**) training group; (**B**) validation group.



Supplementary Figure 2. AUROC of model in COVID-19 diagnosis (**A**), and Calibration chart for predicted versus observed probability (**B**, **C**) in training and validation group.

Supplementary Tables

Supplementary Table 1. Characteristics of patients with SARS-CoV-2 infected.

Characteristic	Development group	Validation group	P-value
Number	59	44	
Female	24 (40.68%)	15 (34.09%)	0.495
Age (years)	47 (38-56)	48 (35-57)	0.812
Symptom			
Fever	37 (62.71%)	32 (72.73%)	0.285
Dry cough	24 (40.68%)	18 (40.91%)	0.981
Fatigue	6 (10.17%)	4 (9.09%)	0.855
Pharyngalgia	14 (23.73%)	8 (18.18%)	0.497
Blood parameters			
Leucocyte (109/L)	5.10 (4.05-6.05)	4.50 (3.63-6.03)	0.738
hsCRP (mg/L)	10.17 (2.62-21.88)	14.80 (5.35-30.10)	0.043
Monocyte (109/L)	0.40 (0.30-0.50)	0.35 (0.27-0.52)	0.224
RBC (1012/L)	4.70 (4.25-5.01)	4.69 (4.22-5.01)	0.88
Hematocrit (%)	0.42 (0.38-0.45)	0.42 (0.38-0.44)	0.668
Lymphocyte (109/L)	1.10 (0.85-1.50)	1.10 (0.73-1.30)	0.134
MCH (pg)	30.60 (29.65-31.30)	30.15 (29.30-31.33)	0.439
MCHC (g/L)	341.00 (334.00-346.50)	341.00 (334.50-348.00)	0.939
MPV	10.40 (9.90-10.85)	10.35 (9.95-11.00)	0.707
Basophilicgranulocyte (109/L)	0.01 (0.01-0.02)	0.01 (0.00-0.01)	0.066
Eosinophil (109/L)	0.01 (0.00-0.03)	0.00 (0.00-0.02)	0.757
Hemoglobin (g/L)	144.00 (129.00-153.00)	143.00 (129.00-152.00)	0.641
PDW (%)	12.00 (11.20-13.10)	11.90 (10.73-13.03)	0.403
Platelet (109/L)	192.00 (144.50-234.00)	177.00 (140.00-226.00)	0.4
Platelet hematocrit (%)	0.20 (0.15-0.23)	0.18 (0.15-0.24)	0.453
Neutrophil (109/L)	3.40 (2.60-4.45)	3.00 (2.18-4.15)	0.981
Radiological evidence of pneumonia	24 (40.68%)	19 (43.18%)	0.799

Abbreviations: HsCRP: high-sensitivity C-reactive proteins; RBC: Red Blood Cell; MCH: mean corpuscular hemoglobin; MPV: mean platelet volume; MCHC: mean corpuscular hemoglobin concentration; PDW: Platelet distribution width; CT: chest computed tomography scan.

Supplementary Table 2. Characteristics of patients visited fever clinics.

Characteristic	Non-COVID-19 infected	COVID-19 infected	*P*-value
Number	452	44	
Female	217 (48.01%)	15 (34.09%)	0.077
Age (years)	31 (25-38)	48 (35-57)	<0.001
Symptom			
Fever	335 (74.12%)	32 (72.73%)	0.841
Dry cough	153 (33.85%)	18 (40.91%)	0.347
Fatigue	39 (8.63%)	4 (9.09%)	0.917
Pharyngalgia	81 (17.92%)	8 (18.18%)	0.966
Blood parameters			
Leucocyte (10^9/L)	7.20 (5.50-9.50)	4.50 (3.63-6.03)	<0.001
hsCRP (mg/L)	8.80 (2.40-22.22)	14.80 (5.35-30.10)	0.547
Monocyte (10^9/L)	0.57 (0.43-0.78)	0.35 (0.27-0.52)	<0.001
RBC (10^{12}/L)	4.74 (4.38-5.14)	4.69 (4.22-5.01)	0.068
Hematocrit (%)	0.42 (0.40-0.46)	0.42 (0.38-0.44)	0.042
Lymphocyte (10^9/L)	1.29 (0.87-1.76)	1.10 (0.73-1.30)	0.004
MCH (pg)	30.30 (29.50-31.20)	30.15 (29.30-31.33)	0.442
MCHC (g/L)	339.00 (332.00-344.00)	341.00 (334.50-348.00)	0.046
MPV	9.95 (9.30-10.60)	10.35 (9.95-11.00)	<0.001
Basophilicgranulocyte (109/L)	0.02 (0.01-0.03)	0.01 (0.00-0.01)	<0.001
Eosinophil (109/L)	0.03 (0.01-0.09)	0.00 (0.00-0.02)	0.002
Hemoglobin (g/L)	144.00 (132.00-156.00)	143.00 (129.00-152.00)	0.177
PDW (%)	11.20 (10.10-12.45)	11.90 (10.73-13.03)	0.15
Platelet (109/L)	214.00 (176.00-260.00)	177.00 (139.75-226.00)	<0.001
Platelet hematocrit (%)	0.22 (0.18-0.25)	0.18 (0.15-0.24)	0.002
Neutrophil (109/L)	4.90 (3.50-7.22)	3.00 (2.18-4.15)	<0.001
Radiological evidence of pneumonia	44 (9.73%)	19 (43.18%)	<0.001

Abbreviations: HsCRP: high-sensitivity C-reactive proteins; RBC: Red Blood Cell; MCH: mean corpuscular hemoglobin; MPV: Mean platelet volume; MCHC: mean corpuscular hemoglobin concentration; PDW: Platelet distribution width; CT: chest computed tomography scan.

Research Paper

COVID-19 induced liver function abnormality associates with age

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) is a novel infectious disease that may cause fever, dry cough, fatigue and shortness of breath. The impact of COVID-19 on liver function is not well described.
Results: We found that the overall frequency of LFT abnormality was 17.6%. Frequency of LFT abnormality was significantly greater in patients with severe/critical (SC) COVID-19 compared to those with mild/moderate (MM) COVID-19 (32.4% vs 11.6%, p=0.011). Among patients with LFT abnormality, the median age was significantly higher in the SC group compared to the MM group (52 vs 39 years, p=0.021).
Conclusion: COVID-19 is frequently associated with mild liver function abnormality, particularly in individuals with severe/critical COVID-19 who were older. Liver function should be monitored carefully during infection, with judicious use of hepatotoxic agents where possible and avoidance of prolonged hypotension to minimize liver injury in older patients.
Methods: The No. 2 People's Hospital of Fuyang City in China has admitted a total of 159 patients with confirmed COVID-19 since the outbreak from January 2020 to March 2020. We analyzed the incidence of liver function test (LFT) abnormality in these patients with confirmed COVID-19 infection.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a novel infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS coronavirus 2 or SARS-CoV-2) [1–5]. The COVID-19 outbreak was first described in November/December 2019 in China, and has since spread to over 180 countries around the world [6–10]. Due to this rapid spread and severity of the illness, the World Health Organization characterized COVID-19 as a pandemic [11–13]. COVID-19 continues to be a serious threat to public health worldwide, with a global morality rate of 5.15% as of June 24th 2020 [10]. However, the mortality rate has varied significantly across regions, ranging from low rates in Qatar (0.11%), Russia (1.40%) and South Africa (1.98%), to intermediate rates in India (3.17%), Germany (4.63%), Iran (4.70%), China (5.48%) and the United States (5.16%), to very high rates in Spain (11.48%), the United Kingdom (13.98%), Italy

(14.52%), France (15.03%) and Belgium (15.97%) (see the Coronavirus Resource Centre or the latest worldwide data [10, 14]). COVID-19 most commonly causes fever, cough, shortness of breath, myalgia, fatigue, and sore throat [1], ranging from mild in severity to severe, with around a quarter requiring intensive care admission in the largest case series to date [1, 15]. Asymptomatic infection with confirmed transmission and atypical presentations with abdominal pain, nausea, vomiting and diarrhea have also been reported [15]. However, the frequency of liver dysfunction in COVID-19 infection has not been well described, and in particular have been difficult to interpret due to co-administration of hepatotoxic agents and varied timing of liver function abnormality in the course of the illness and across age groups [16–18]. In this brief report, we sought to analyze the association between COVID-19 infection, liver function test (LFT) abnormality and age in the 159 patients hospitalized for confirmed COVID-19 at the No. 2 People's Hospital of Fuyang City, Fuyang, Anhui Province, China.

RESULTS

Patients

A total of 159 patients were admitted with confirmed COVID-19 and enrolled in this study. Baseline demographics and patient characteristics according to severity of COVID-19 are presented in Table 1. Overall, the median age was 43 years, and 56.6% (90/159) were male (Table 1). Thirty-four patients (21.4%) were classified to have severe or critical illness (SC) and the remaining 125 patients (78.6%) were classified to have mild/moderate illness (MM) (Tables 1, 2). In brief, patients in the MM group were significantly younger, had a lower body mass index (BMI), were less likely to have fever, and had a lower heart rate, lower respiratory rate and higher oxygen saturations at admission compared to the SC group (Table 1), reflecting the severity of their COVID-19. There was a significantly higher proportion of patients with underlying chronic hepatitis B virus (HBV) infection in the SC group compared to the MM group (Table 1). There was a significantly higher proportion of patients with hypertension in the SC group compared to the MM group (Table 1). Other comorbidities were similar between both groups. Patients with chronic HBV were treated with entecavir (ETV) if they met the APASL guidelines for HBV treatment [23].

Liver function test abnormality frequency

Twenty-eight of the 159 (17.6%) hospitalized patients had LFT abnormality at the time of hospital admission (n=19), and a further 9 patients (5.7%) developed LFT abnormality during the first week of admission. The proportion of patients with LFT abnormality was significantly higher in the SC group compared to the MM group (32.4% vs 11.6%, p=0.011, Table 1).

Among patients with LFT abnormality, the median age was significantly higher in the SC group compared to the MM group (52 vs 39 years, p=0.021). Three patients had a history of chronic HBV infection, 2 of whom were receiving antiviral therapy with ETV (Tables 2, 3). The distribution of comorbidities was similar among the subset of patients with liver function test abnormality in the MM and SC groups, and in particular there was a similar number of patients with chronic HBV and patients receiving ETV in each group (Tables 2, 3 and Figure 1).

Pattern and degree of liver function test abnormality

We analyzed the components of the LFT panel, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are markers of hepatocellular damage, and gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP), which are markers of cholestasis, in COVID-19 patients at the time admission (week 0), at 1, 2 and 6 weeks following date of admission as an inpatient or outpatient depending on length of admission. In addition, we analyzed markers of liver synthetic function including total bilirubin (TBIL), and coagulation profiles using the international normalized ratio (INR). We found that there was only a mild to moderate derangement in LFTs in both MM and SC patient groups (Tables 3, 4 and Figures 1, 2), with a mixed pattern of both hepatocellular injury and cholestasis (GGT elevations observed but no changes in ALP were observed), without significant liver synthetic dysfunction.

In more detail, the majority of patients had ALT, AST and GGT levels below 5 times the ULN (Table 4 and Figure 2). In the SC group, 4 (36.4%) patients had an ALT 1-2x ULN, 4 (36.4%) patients had an ALT 2-5x ULN, and 3 (27.3%) patients had an elevated ALT >5x ULN. In the MM group, 9 (52.9%) patients had an ALT 1-2x ULN, 7 (41.2%) patients had an ALT 2-5x ULN, and 1 (5.9%) patient had an ALT >5x ULN. AST was abnormal in 8 SC patients and 7 MM patients with LFT abnormalities. In the SC group, 4 (50%) patients had an AST of 1-2x ULN, 2 (25%) patients had AST 2-5x ULN, and 2 (25%) patients had an AST greater than 5 ULN (Table 4 and Figure 2). A total of 7 (100%) patients had an AST 1-2x ULN in the MM group, and no elevations >2x ULN were noted in this group (Table 4 and Figure 2). Median ALT and AST values failed to reach statistical significance between the SC and MM groups (Table 4). GGT levels were abnormal in 10 SC

Table 1. Comparison of baseline demographics and clinical characteristics between SC and MM groups. Values are expressed as median (interquartile range (IQR), 25-75%). P value is the comparison between severe/critical (SC) and mild/moderate (MM) patients. *P<0.05, **P<0.01, ***P<0.001.

Characteristic	SC	MM	P value
Total number (n, %)	34 (21.4%)	125 (78.6%)	
with liver function test abnormality (n, %)	11 (32.4%)	17 (11.6%)	0.011
Age (years) (median, IQR)	49.5 (42.5-65.3)	41.0 (29.0-50.0)	<0.0001
Male gender (n, %)	23 (67.6%)	67 (53.6%)	0.143
Fever (n, %)	34(100%)	84(67.2%)	<0.0001
Temperature (°C) (Median, IQR)	37.1 (36.8-37.9)	36.8 (36.5-37.5)	0.014
Heart rate (beats / minute) (Median, IQR)	96 (78-102)	84 (80-91)	0.039
Blood Pressure (mmHg) (Median, IQR)	130 (116-142)/ 84 (73-93)	128 (119.5-140)/ 85 (75.5-92)	0.671/ 0.711
Respiratory rate (breaths / minute) (Median, IQR)	20 (19-23)	20 (19-21.5)	0.031
Oxygen saturation (%) (Median, IQR)	91.5(89.5-94.3)	98(97-98)	<0.0001
Treatment with lopinavir/ritonavir (n, %)	30(88.2%)	109(87.2%)	0.798
Treatment with lopinavir/ritonavir and hydroxychloroquine (n, %)	1(2.9%)	15(12%)	0.07
Body mass index (kg/m^2) (Median, IQR)	25.8 (23.4-27.6)	24.2 (22.1-26.1)	0.022
Comorbidities	**SC**	**MM**	**P value**
Chronic hepatitis B virus (HBV)	9	3	<0.0001
Chronic HBV receiving entecavir	1	2	0.517
HBV-related cirrhosis	0	0	a/n
HBV cirrhosis	0	0	a/n
Hypertension	13	11	<0.0001
Diabetes	4	10	0.492
Coronary heart disease	3	0	0.009
Fatty liver	1	1	0.321
Other	9	1	<0.0001

Table 2. Comparison of baseline demographics and clinical characteristics between SC and MM groups in the subset with liver function test abnormality.

Characteristic	SC	MM	P value
Number (n, %)	11 (32.4%)	17 (11.6%)	0.011
Age (years) (Median, IQR)	52 (40-63)	39 (30-47.0)	0.021
Male gender (n, %)	9 (81.8%)	11 (64.7%)	0.328
Body mass index (kg/m^2) (Median, IQR)	26.2 (25.7-27.0)	24.5 (22.9-26.1)	0.120
Comorbidities	**SC**	**MM**	**P value**
Chronic hepatitis B virus	2 (1 on ETV)	1 (1 on ETV)	0.543
Hepatitis B virus related cirrhosis	0	0	a/n
Hypertension	3	1	0.269
Diabetes	1	1	1
Other	2	0	0.146
The number of comorbidities	**SC**	**MM**	**P value**
One	1	3	1
Two	3	0	0.05
Three	1	0	0.393

Values are expressed as median (interquartile range (IQR), 25-75%). P value is the comparison between severe/critical (SC) and mild/moderate (MM) patients. *P<0.05. ETV = entecavir.

Table 3. Comparison of liver function test parameters between MM and SC group patients with abnormal liver function tests (Table 3-1) and normal liver function tests (Table 3-2).

Table 3-1. Patients with abnormal liver function within 1 week of admission.

		Week 0			Week 1			Week 2			Week 6		
	NRR	SC n=11	MM n=17	P value	SC n=11	MM n=17	P value	SC n=11	MM n=17	P value	SC n=11	MM n=17	P value
ALT (U/L)	0-50	69 (27-81)	62 (33.0-90.5)	0.962	70 (49-119)	60 (49-106)	0.64	47 (25-210)	41.0 (22-71.5)	0.378	25.5 (15.5-45.5)	42.5 (20.3-49.5)	0.291
AST (U/L)	0-50	46 (30-65)	40 (28-52.5)	0.423	37.50 (25.25-74.0)	24.0 (18.0-44.25)	0.078	34.50 (24.25-38.5)	25.0 (21.0-31.5)	0.128	21.5 (17.8-25.8)	23.5 (20-29)	0.178
GGT (U/L)	10-60	59 (21-130)	35 (21-108.5)	0.48	96 (55-114)	48 (24-99)	0.082	66 (41.5-166.5)	40 (22.5-79)	0.217	49.5 (23-87.5)	34 (22.3-73.5)	0.752
ALP (U/L)	45-125	62 (48-75)	64 (55-72)	0.495	56 (48-63)	61 (51-79)	0.232	58 (49-64)	67 (47-92.4)	0.045	66.5 (57.8-78.3)	71 (58.8-87)	0.598
TBIL (µmmol/L)	0-26	13.40 (8.6-33.1)	11.5 (7.2-15.5)	0.279	13.4 (8.6-33.1)	11.8 (8.5-15.3)	0.264	10.9 (7.8-18.4)	7.6 (6.3-12.6)	0.115	9.4 (5.6-16.9)	12 (9.5-13.5)	0.562
INR	0.94-1.30	1 (0.94-1.11)	0.98 (0.91-1.12)	0.925	0.93 (0.85-0.98)	0.92 (0.85-0.95)	0.744	0.92 (0.9-0.99)	0.88 (0.87-0.93)	0.176	0.93 (0.88-0.97)	0.91 (0.88-0.95)	0.735

Normal reference range (NRR), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBIL), and international normalized ratio (INR).

Table 3-2. Patients with normal liver function within 1 week of admission.

		Week 0			Week 1			Week 2			Week 6		
	NRR	SC n=23	MM n=108	P	SC n=23	MM n=108	P	SC n=23	MM n=108	P	SC n=23	MM n=108	P
ALT (U/L)	0-50	25 (18-33)	23 (13-36)	0.515	22 (18-32)	19 (13-34.5)	0.632	30 (22-47.5)	28.5 (15-44)	0.206	18 (13.5-33.8)	26 (15-40)	0.25
AST (U/L)	0-50	28 (23-30)	25 (19-31)	0.091	24 (19-26)	21 (18-24)	0.197	21 (18-28.5)	20 (16-26)	0.394	19 (16-26)	23 (18-30)	0.07
GGT (U/L)	10-60	23 (16-33)	26 (15-41)	0.934	23 (18-29)	22 (15-38)	0.55	30 (21-46.5)	26.5 (16.8-53)	0.416	21 (15.5-26.8)	29 (17-46)	0.099
ALP (U/L)	45-125	61 (46-66)	63 (51-73)	0.17	52 (45-58)	59 (48.5-70)	0.006	54 (40-65)	59 (51-72.3)	0.064	61 (51.8-74)	62 (53-77)	0.602
TBIL (µmmol/L)	0-26	11.4 (7.2-15.2)	9.9 (7.0-15.3)	0.495	13.6 (8.1-17.7)	11.9 (9.4-15.6)	0.951	9.8 (6.4-19.3)	7.5 (5.9-10.2)	0.034	11 (6.5-13)	10.5 (8.4-14.1)	0.562
INR	0.94-1.30	1.01 (0.95-1.07)	0.98 (0.94-1.07)	0.702	0.95 (0.93-1.01)	0.93 (0.88-1.0)	0.123	0.95 (0.88-1.08)	0.93 (0.9-0.95)	0.505	0.91 (0.87-0.97)	0.91 (0.89-0.92)	0.91

Values are expressed as median (interquartile range (IQR), 25-75%). P value is the comparison between Severe, Critical (SC) and Mild, Moderate (MM) group patients.

patients and 17 MM patients with LFT abnormalities (Table 4). In the SC group, 6 (60%) patients had elevated GGT levels 1-2x ULN, and 4 (40%) patients had elevated GGT levels 2-5x ULN. In the MM group, 14 (82.4%) patients had elevated GGT levels 1-2x ULN, and 3 (17.6%) patients had elevated GGT levels 2-5x ULN (Table 4 and Figure 2). Only 1 patient had an abnormal ALP in MM group (1-2x ULN), and no patients had abnormal ALP levels in the SC group (Table 4 and Figure 2). Only 3 patients had an elevated TBIL in the SC group; 1 patient had a TBIL 1-2x ULN, and 2 patients had a TBIL 2-5x ULN (all 3 patients had an elevated ALT, but normal ALP and INR) (Table 4 and Figure 2). TBIL elevation was not observed in the MM group. All patients had a normal INR (Tables 3, 4 and Figures 1, 2). Patients who experienced elevations in ALT above 2x ULN received glycyrrhizin therapy, which is routinely used as a hepatoprotective agent in our institution. LFT abnormalities recovered in all patients, and median time to normalization was 10 days.

In our case series, the most significantly elevated ALT was observed in a patient with chronic HBV who was treatment-naïve, where the peak ALT was 414 U/L, with an AST of 309 U/L, GGT of 290 U/L, ALP of 86 U/L, and an elevated TBIL of 70.5 µmol/L, but normal INR (1.08). Further characterization of the HBV revealed the patient was HBsAg positive, HBeAg positive and the HBV DNA was elevated at 22,800 IU/mL. Given the significant elevation in HBV DNA,

the treating clinician felt that the LFT abnormalities were more likely attributable to their chronic HBV rather than COVID-19, and entecavir was commenced, in addition to glycyrrhizin therapy for 13 days. The LFTs normalized over the following 13 days in this patient.

DISCUSSION

COVID-19 can lead to symptoms including fever, cough, fatigue, shortness of breath and myalgias. In more severe disease, COVID-19 may cause significant shortness of breath, hypoxia and respiratory failure, as well as radiographic features of pneumonia and/or other lung infiltrates. Although the lung is the primary target organ of SARS-CoV-2, confirmed at autopsy and characterized by an inflammatory reaction in the deep airway and alveolar injury [24], there are several reports that COVID-19 may also cause liver function test abnormality [1, 16, 17, 25], however these case series are difficult to interpret due to frequent co-administration of hepatotoxic agents such as lopinavir /ritonavir and other conditions that may lead to liver injury such as ischaemic hepatitis from severe and/or prolonged hypotension/shock. In this report, we observed that LFT abnormality is frequent in COVID-19

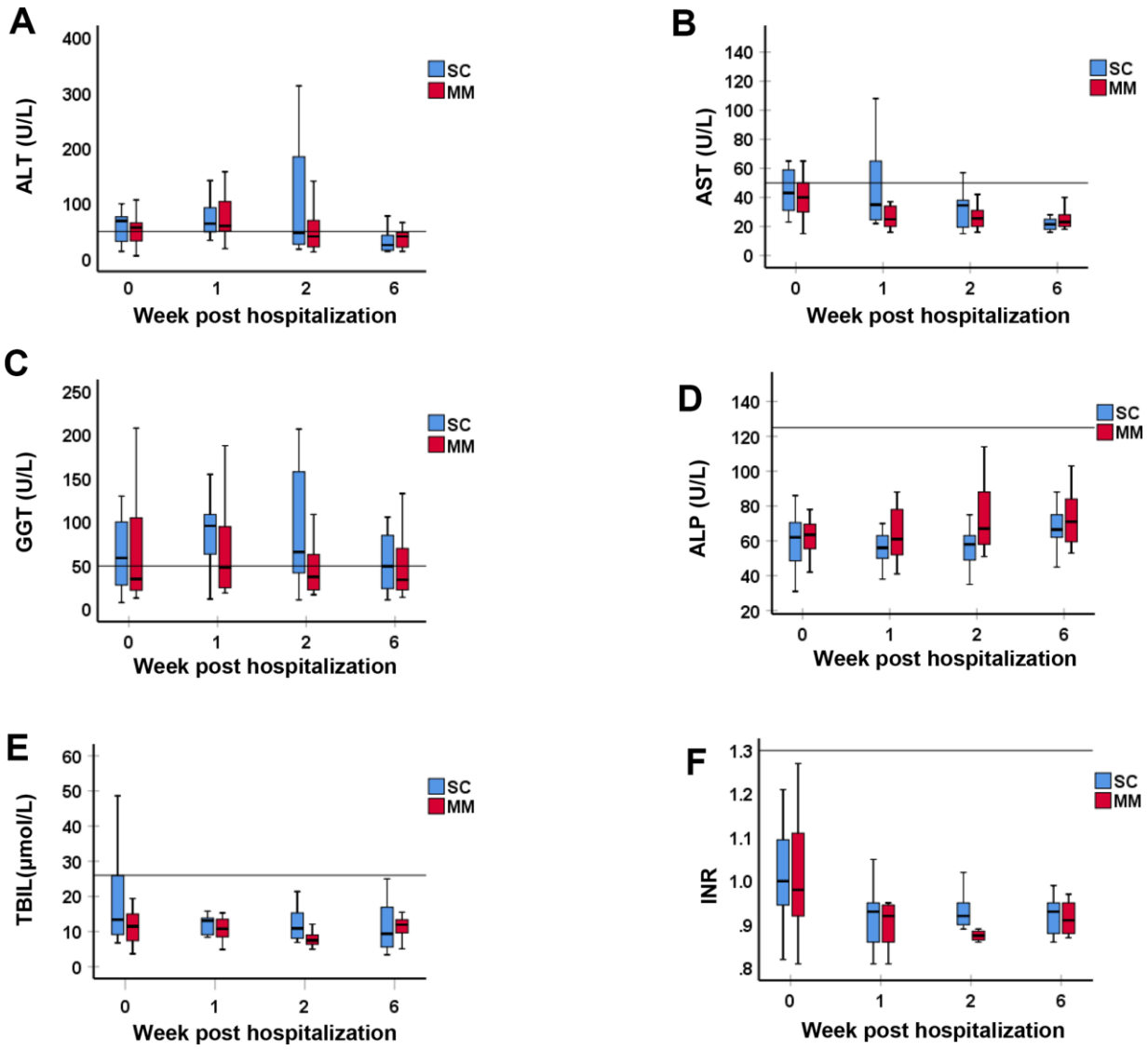

Figure 1. Comparison of liver function test between MM and SC patient groups with liver function test abnormality. The liver function tests including (**A**) ALT, (**B**) AST, (**C**) GGT, (**D**) ALP, (**E**) TBIL, and (**F**) INR, were compared between MM and SC patient groups with liver function test abnormality at Week 0, 1, 2 and 6 post hospitalization for COVID-19. Values are expressed as median (interquartile range (IQR), 25-75%). The horizontal line in each panel is the upper limit of normal (ULN) for each parameter. There was no statistically significant difference in any of the LFT or INR parameters between SC and MM patients.

Table 4. Degree of liver function test abnormality in SC and MM groups in the subset with liver abnormality.

	1-2 ULN		P value	2-5 ULN		P value	> 5ULN		P value
	SC	MM		SC	MM		SC	MM	
ALT (n, %)	4(11,36.4%)	9(17,52.9%)	0.057	4(11,36.4%)	7(17, 41.2%)	0.799	3(11,27.3%)	1(17,5.9%)	0.269
AST (n, %)	4(8,50%)	7(7,100%)	0.799	2(8,25%)	0	0.543	2(8,25%)	0	0.146
GGT (n, %)	6(10,60%)	14(17,82.4%)	0.112	4(10,40%)	3(17,17.6%)	0.264	0	0	a/n
ALP (n, %)	0	1(100%)	0.206	0	0	a/n	**0**	**0**	a/n
TBIL (n, %)	1(3,33.3%)	0	0.206	2(3,66.7%)	0	0.068	0	0	a/n
INR	0	0	a/n	0	0	a/n	0	0	a/n

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBIL), and international normalized ratio (INR).
Values are expressed as median (interquartile range (IQR), 25-75%). P value is the comparison between severe/critical (SC) and mild/moderate (MM) patients.

patients hospitalized at the No. 2 People's Hospital of Fuyang City, with an overall frequency of LFT abnormality of 17.6% in the 159 patients with confirmed COVID-19. In addition, our study demonstrates that older patients are more likely to develop more severe COVID-19, which has been observed throughout the world, and are also more likely to develop LFT abnormality [18]. Furthermore, we observed a significantly higher proportion of patients with liver function test abnormality in the SC group compared to the MM group, suggesting a greater frequency of liver dysfunction in the SC group. These findings are consistent with other reports of COVID-19 in China [15, 25], and importantly demonstrates frequent LFT abnormality prior to the administration of potentially hepatotoxic agents. Our study therefore offers some interesting findings of liver involvement during COVID-19 infection.

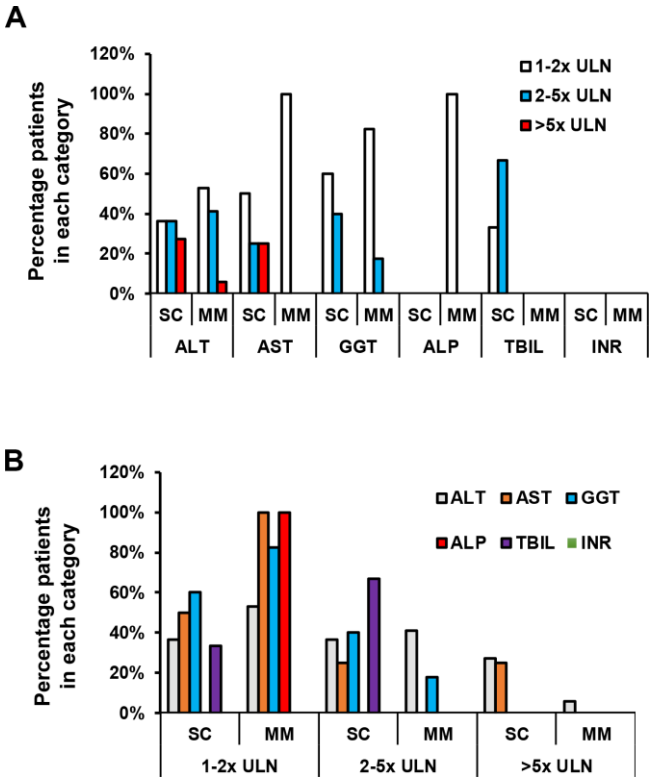

Figure 2. Degree of liver function test abnormality in SC and MM groups in the subset with liver function test abnormality. (**A**) Comparison of liver function abnormality between SC and MM groups with liver abnormality. (**B**) Comparison of liver function subset between SC and MM groups with liver abnormality. There was no statistically significant difference in the degree of LFT abnormality between SC and MM patients.

Liver dysfunction has been seen during other respiratory virus pandemics, although the incidence of LFT dysfunction was more severe with pandemic A/H1N1 influenza in 2009 than during this current COVID-19 outbreak [26], whereby serum levels of AST, ALT, and GGT were significantly higher in the A/H1N1 influenza than observed in COVID-19. Interestingly, abnormalities in serum liver enzymes were strongly correlated with hypoxemia in the A/H1N1 influenza pandemic, suggesting that influenza itself may in some way mediate the hepatotoxicity [26]. When comparing liver function test parameters between our SC and MM COVID-19 patients, we found that the SC patients had a higher incidence of liver injury to MM patients, however the pattern and degree of LFT abnormality was not significantly different between the two groups with regards to the proportion of patients with LFT abnormalities 1-2x ULN, 2-5x ULN and >5x ULN. We did observe that median ALT and AST values were numerically higher in SC patients compared to MM patients, with median ALT values above the ULN in the SC group, however this failed to reach statistical significance. These findings indicate that COVID-19 is associated with mild to moderate liver function test abnormalities with a mixed picture of liver injury,

particularly in SC patients. However, accompanying significant liver synthetic function compromise or liver failure were not observed in this cohort. In addition, our findings indicate that older patients are not only more likely to develop more severe COVID-19 but are also at greater risk of liver function abnormality.

It should be noted that the vast majority of the patients enrolled in this study received lopinavir/ritonavir with or without hydroxychloroquine as potential antiviral agents. Lopinavir/ritonavir is a well described to cause drug-induced liver injury (DILI). Therefore, we designed our study to restrict the definition of LFT abnormality to the first week following admission in order to limit potential confounding from hepatotoxicity from lopinavir/ritonavir. However, liver function parameters did not significantly change from baseline or week 1 to week 2, indicating that lopinavir/ritonavir-induced DILI does not explain our findings.

There are increasing reports of COVID-19 induced liver dysfunction in China, where mild elevations in liver functions tests have also been described [1, 15, 27, 28]. However, the mechanism by which COVID-19 induces liver function abnormality is not well characterized. There is much speculation regarding potential mechanisms, which include direct liver injury from SARS-CoV-2 infection of hepatocytes, cytokine storm syndrome, DILI and ischaemic hepatitis. We speculate that hepatocytes could be infected given SARS caused by SARS-CoV-1, another coronavirus similar to SARS-CoV-2, as SARS-CoV-1 RNA was detected in liver tissue from patients with SARS, although viral inclusions were not seen on electron microscopy [29].

Interestingly, non-specific histological features of microvascular steatosis and mild lobular and portal activity has been observed in the liver at autopsy in a patient who died of severe COVID-19 [27]. However, viral inclusions were not identified in liver tissue at autopsy and therefore it is unclear if these changes were related to direct viral infection of the liver by SARS-CoV-2, DILI, or even due to pre-existing fatty liver disease, although it should be noted that viral inclusions were also not seen in lung tissue (the primary target organ of COVID-19) in this patient. Another potential mechanism that has been considered is the effect of COVID-19 induced cytokine storm syndrome (CSS) on liver injury, but without strong evidence supporting this hypothesis. Liver damage may also be influenced by underlying liver diseases, such as chronic HBV and fatty liver disease, or as a result of pneumonia-associated hypoxia or ischaemic hepatitis from prolonged hypotension. These data highlight that further studies are required to elucidate the mechanism(s) of liver impairment in COVID-19.

In summary, we found that COVID-19 associated liver function test abnormality is more common in patients with severe or critical presentations of COVID-19, as well as older patients. Although the degree of COVID-19 induced liver function abnormality is relatively mild to moderate in our cohort without evidence of significant liver synthetic dysfunction or liver failure, it highlights the frequent incidence of LFT abnormalities in patients with COVID-19, which has implications for the management of these patients in order to preserve liver function with consideration of co-administration of hepatoprotective agents and to minimize exposure to hepatotoxic events, particularly in patients with underlying liver disease and older age. Our study adds to the growing body of evidence that SARS-CoV-2 is associated with liver function test abnormality, and particularly in older patients [18, 30, 31]. A more detailed understanding of the underlying mechanisms of liver injury from SAR-CoV-2, as well as viral pathogenesis and antiviral responses to COVID-19 are therefore required in order to best optimize older age patient outcomes.

MATERIALS AND METHODS

Patients and study design

As of March 4th, 2020, the No. 2 People's Hospital of Fuyang City has admitted 159 patients (including 4 patients transferred from Bozhou City, Anhui Province) with confirmed COVID-19 since the outbreak of the disease in Anhui Province in January 2020. No COVID-19 related deaths have been recorded in this hospital. The majority of the patients enrolled in this study received lopinavir/ritonavir with or without hydroxychloroquine for antiviral therapy. All COVID-19 patients were diagnosed, classified and treated according to the guidelines of the Pneumonia Treatment Plan for the Novel Coronavirus Infection, National Health and Health Commission of the people's Republic of China (Version 1-6) [19–22]. Patients with confirmed COVID-19 were included, and classification of severity criteria was as follows: 1) mild: mild clinical symptoms without pneumonia on imaging; 2) moderate: fever, respiratory tract infection symptoms with pneumonia on imaging; 3) severe: confirmed COVID-19 with one or more of the following 3 features: (a) breathing distress, respiratory rate ≥ 30 breaths/minute, (b) oxygen saturation ≤ 93% on room air, or (c) oxygenation index ≤ 300mmHg; 4) critical: confirmed COVID-19 with one or more of the following 3 features: (a) respiratory failure requiring mechanical ventilation, (b) coma, (c) combined organ failure

requiring ICU monitoring (for example, dysfunction/ failure of more than 2 organ systems that requires ICU support). Exclusion criteria included patients with respiratory symptoms that repeatedly tested negative for COVID-19 and did not have pneumonia on imaging, and those with new onset of liver dysfunction one week after hospitalization. This time point was chosen in order to exclude patients that may have developed abnormal LFTs from another cause such as ischaemic hepatitis from prolonged hypotension/shock or drug-induced liver injury. In this report, we sought to analyze the frequency of LFT abnormality and liver dysfunction in COVID-19 patients, and specifically to compare the incidence of LFT abnormality and liver dysfunction between COVID-19 patients with mild or moderate illness (MM group) and those with severe or critical illness (SC group). LFT abnormality is defined as any parameter of the liver enzyme panel greater than the upper limit of normal (ULN). We also evaluated liver synthetic function abnormality with International Normalized Ratio (INR) and elevated total bilirubin (TBIL).

Ethics approval and consent to participate

This study was approved by the Ethics Review Committee of the No. 2 People's Hospital of Fuyang City (reference number: 2020006) and was conducted in accordance with the ethical standards of the institutional and national research committees, and with the 1964 declaration of Helsinki. This study was registered at the Chinese Clinical Trial Registry (registration number ChiCTR2000031620).

COVID-19 detection and laboratory parameter testing

Nasopharyngeal aspirates and sputum from patients with suspected COVID-19 were used for COVID-19 testing. COVID-19 RNA was detected by using real-time quantitative PCR (qPCR) (Shanghai BioGerm Medical Biotechnology Co., Shanghai, China). Liver function was measured by using the Hitachi 7600 fully automatic biochemical analyzer. The complete blood count was measured by using the SYSMEX CA5100 automatic clotting analyzer (Siemens Healthcare, Erlangen, Germany). Internationalized Normalized Ratio (INR) was calculated based on the prothrombin time (PT) test result.

Statistical analyses

Data were analyzed using SPSS Statistics v25.0 (Armonk, New York, USA). Continuous data were expressed as medians with interquartile range, and categorical data as frequencies. Groups were compared using the Mann-Whitney U test, and the correlations between clinical, laboratory parameter were evaluated using the two-tailed chi-squared test.

AUTHOR CONTRIBUTIONS

Shasha Li, Jinsong Li, Tuo Shao, Xiuyong Li, Jacinta A. Holmes, Wenyu Lin and Mingfeng Han designed the research study, analyzed and interpreted the data, and wrote the manuscript. Shasha Li, Jinsong Li, Zhenhua Zhang, Lin Tan, Ming Li, Xiuyong Li and Mingfeng Han were involved in diagnosis and treatment of patients, recruiting patients and collection of clinical data. All authors were involved in critical appraisal of the manuscript and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors have declared that no conflicts of interest exist.

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REFERENCES

1. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

2. Menachery VD, Schäfer A, Burnum-Johnson KE, Mitchell HD, Eisfeld AJ, Walters KB, Nicora CD, Purvine SO, Casey CP, Monroe ME, Weitz KK, Stratton KG, Webb-Robertson BM, et al. MERS-CoV and H5N1 influenza virus antagonize antigen presentation by altering the epigenetic landscape. Proc Natl Acad Sci USA. 2018; 115:E1012–21. https://doi.org/10.1073/pnas.1706928115 PMID:29339515

3. Song Z, Xu Y, Bao L, Zhang L, Yu P, Qu Y, Zhu H, Zhao W, Han Y, Qin C. From SARS to MERS, thrusting coronaviruses into the spotlight. Viruses. 2019; 11:59. https://doi.org/10.3390/v11010059 PMID:30646565

4. The Lancet. Emerging understandings of 2019-nCoV. Lancet. 2020; 395:311.

https://doi.org/10.1016/S0140-6736(20)30186-0
PMID:31986259

5. Yin Y, Wunderink RG. MERS, SARS and other coronaviruses as causes of pneumonia. Respirology. 2018; 23:130–37. https://doi.org/10.1111/resp.13196 PMID:29052924

6. Ki M, and Task Force for 2019-nCoV. Epidemiologic characteristics of early cases with 2019 novel coronavirus (2019-nCoV) disease in Korea. Epidemiol Health. 2020; 42:e2020007. https://doi.org/10.4178/epih.e2020007 PMID:32035431

7. Giovanetti M, Benvenuto D, Angeletti S, Ciccozzi M. The first two cases of 2019-nCoV in Italy: where they come from? J Med Virol. 2020; 92:518–21. https://doi.org/10.1002/jmv.25699 PMID:32022275

8. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, Xing F, Liu J, Yip CC, Poon RW, Tsoi HW, Lo SK, Chan KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet. 2020; 395:514–23. https://doi.org/10.1016/S0140-6736(20)30154-9 PMID:31986261

9. Lu H, Stratton CW, Tang YW. Outbreak of pneumonia of unknown etiology in Wuhan, China: the mystery and the miracle. J Med Virol. 2020; 92:401–02. https://doi.org/10.1002/jmv.25678 PMID:31950516

10. Liu RB, Tayal VS, Panebianco NL, Tung-Chen Y, Nagdev A, Shah S, Pivetta E, Henwood PC, Nelson MJ, Moore CL. Ultrasound on the frontlines of COVID-19: report from an international webinar. Acad Emerg Med. 2020; 27:523–26. https://doi.org/10.1111/acem.14004 PMID:32348585

11. Hui DS, I Azhar E, Madani TA, Ntoumi F, Kock R, Dar O, Ippolito G, Mchugh TD, Memish ZA, Drosten C, Zumla A, Petersen E. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health - the latest 2019 novel coronavirus outbreak in Wuhan, China. Int J Infect Dis. 2020; 91:264–66. https://doi.org/10.1016/j.ijid.2020.01.009 PMID:31953166

12. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, Ren R, Leung KS, Lau EH, Wong JY, Xing X, Xiang N, Wu Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med. 2020; 382:1199–207. https://doi.org/10.1056/NEJMoa2001316 PMID:31995857

13. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

14. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. Lancet Infect Dis. 2020; 20:533–34. https://doi.org/10.1016/S1473-3099(20)30120-1 PMID:32087114

15. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

16. Cai Q, Huang D, Yu H, Zhu Z, Xia Z, Su Y, Li Z, Zhou G, Gou J, Qu J, Sun Y, Liu Y, He Q, et al. COVID-19: abnormal liver function tests. J Hepatol. 2020; S0168-8278:30218. https://doi.org/10.1016/j.jhep.2020.04.006 PMID:32298767

17. Zhang C, Shi L, Wang FS. Liver injury in COVID-19: management and challenges. Lancet Gastroenterol Hepatol. 2020; 5:428–30. https://doi.org/10.1016/S2468-1253(20)30057-1 PMID:32145190

18. Sargiacomo C, Sotgia F, Lisanti MP. COVID-19 and chronological aging: senolytics and other anti-aging drugs for the treatment or prevention of corona virus infection? Aging (Albany NY). 2020; 12:6511–17. https://doi.org/10.18632/aging.103001 PMID:32229706

19. Novel coronavirus pneumonia diagnosis and treatment plan (Trial Version Sixth). National Health and Health Commission of the people's Republic of China. 2020; http://www.nhc.gov.cn/yzygj/s7653p/202002/8334a8326dd94d329df351d7da8aefc2/files/b218cfeb1bc54639af227f922bf6b817.pdf.

20. Novel coronavirus pneumonia diagnosis and treatment plan (Version Fourth). National Health and Health Commission of the people's Republic of China. 2020; http://www.nhc.gov.cn/jkj/s3577/202002/573340613ab243b3a7f61df260551dd4/files/c791e5a7ea5149f680fdcb34dac0f54e.pdf.

21. Novel coronavirus pneumonia diagnosis and treatment plan (Version Third). National Health and Health Commission of the People's Republic of China. 2020;

http://www.nhc.gov.cn/jkj/s7923/202001/470b128513fe46f086d79667db9f76a5/files/8faa1b85841f42e8a0febbea3d8b9cb2.pdf.

22. Novel coronavirus pneumonia diagnosis and treatment plan (Revised Version Fifth). National Health and Health Commission of the people's Republic of China. 2020: http://www.nhc.gov.cn/yzygj/s7653p/202002/d202004b895337e219445f202008d202728fcaf202001e202003e202013a.shtml.

23. Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, Chen HL, Chen PJ, Chien RN, Dokmeci AK, Gane E, Hou JL, et al. Asian-pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int. 2016; 10:1–98. https://doi.org/10.1007/s12072-015-9675-4 PMID:26563120

24. Liu Q, Wang R, Qu G, Wang Y, Liu P, Fei G, Ren L, Zhou Y, Liu L. General observation report on the autopsy system of death of new coronavirus pneumonia. Journal of Forensic Medicine, Chinese. 2020; 36:19–21.

25. Liu C, Jiang ZC, Shao CX, Zhang HG, Yue HM, Chen ZH, Ma BY, Liu WY, Huang HH, Yang J, Wang Y, Liu HY, Xu D, et al. [Preliminary study of the relationship between novel coronavirus pneumonia and liver function damage: a multicenter study]. Zhonghua Gan Zang Bing Za Zhi. 2020; 28:107–11. https://doi.org/10.3760/cma.j.issn.1007-3418.2020.02.003 PMID:32077660

26. Papic N, Pangercic A, Vargovic M, Barsic B, Vince A, Kuzman I. Liver involvement during influenza infection: perspective on the 2009 influenza pandemic. Influenza Other Respir Viruses. 2012; 6:e2–5. https://doi.org/10.1111/j.1750-2659.2011.00287.x PMID:21951624

27. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, Liu S, Zhao P, Liu H, Zhu L, Tai Y, Bai C, Gao T, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. Lancet Respir Med. 2020; 8:420–22. https://doi.org/10.1016/S2213-2600(20)30076-X PMID:32085846

28. Guan GW, Gao L, Wang JW, Wen XJ, Mao TH, Peng SW, Zhang T, Chen XM, Lu FM. [Exploring the mechanism of liver enzyme abnormalities in patients with novel coronavirus-infected pneumonia]. Zhonghua Gan Zang Bing Za Zhi. 2020; 28:100–06. https://doi.org/10.3760/cma.j.issn.1007-3418.2020.02.002 PMID:32077659

29. Chau TN, Lee KC, Yao H, Tsang TY, Chow TC, Yeung YC, Choi KW, Tso YK, Lau T, Lai ST, Lai CL. SARS-associated viral hepatitis caused by a novel coronavirus: report of three cases. Hepatology. 2004; 39:302–10. https://doi.org/10.1002/hep.20111 PMID:14767982

30. Zhang L, Pang R, Xue X, Bao J, Ye S, Dai Y, Zheng Y, Fu Q, Hu Z, Yi Y. anti-SARS-CoV-2 virus antibody levels in convalescent plasma of six donors who have recovered from COVID-19. Aging (Albany NY). 2020; 12:6536–42. https://doi.org/10.18632/aging.103102 PMID:32320384

31. Yang J, Li H, Hu S, Zhou Y. ACE2 correlated with immune infiltration serves as a prognostic biomarker in endometrial carcinoma and renal papillary cell carcinoma: implication for COVID-19. Aging (Albany NY). 2020; 12:6518–35. https://doi.org/10.18632/aging.103100 PMID:32339157

Research Paper

COVID-19 mortality in Lombardy: the vulnerability of the oldest old and the resilience of male centenarians

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ABSTRACT

Italy was the first European nation to be affected by COVID-19. The biggest cluster of cases occurred in Lombardy, the most populous Italian region, and elderly men were the population hit in the hardest way. Besides its high infectivity, COVID-19 causes a severe cytokine storm and old people, especially those with comorbidities, appear to be the most vulnerable, presumably in connection to inflammaging. In centenarians inflammaging is much lower than predicted by their chronological age and females, presenting survival advantage in almost all centenarian populations, outnumber males, a phenomenon particularly evident in Northern Italy. Within this scenario, we wondered if: a) the COVID-19 mortality in centenarians was lower than that in people aged between 50 and 80 and b) the mortality from COVID-19 in nonagenarians and centenarians highlighted gender differences.

We checked COVID-19-related vulnerability/mortality at the peak of infection (March 2020), using data on total deaths (i.e. not only confirmed COVID-19 cases). Our conclusion is that excess mortality increases steadily up to very old ages and at the same time men older than 90 years become relatively more resilient than age-matched females.

INTRODUCTION

Italy was the first European country to suffer from the COVID-19 pandemic. A main characteristic of this pandemic, besides high infectivity of its causative agent, is a cytokine storm characterized by an IL-6 centered response [1, 2] and the uneven distribution of severity and mortality among different age classes. Indeed, old people, and particularly those with one or more comorbidity, appear to be the most vulnerable [3, 4]. The reasons of such a high vulnerability to COVID-19 is poorly understood but it has been suggested that a major role is played by inflammaging [5–7], i.e. the low-grade chronic inflammation that is a major driver of aging [8] and whose basic underlying mechanisms are shared with those responsible for frailty and age-related diseases (ARDs), including cardiovascular disease, type 2 diabetes, chronic obstructive pulmonary disease, chronic kidney disease and dementia, among others [9–11]. However, a major

characteristic of old people is their heterogeneity regarding not only their health status (presence/absence of comorbidities, frailty, cognitive status) but also, in particular, their different capability to mount an immune response to pathogens and vaccines [12, 13]. At present it is possible to quantify such heterogeneity using a variety of proteomic [15] and epigenetic biomarkers [16] capable of distinguishing between chronological and biological age, and to predict the risk of developing major ARDs. In particular, we showed that centenarians, i.e. subjects who avoided or largely postponed all major ARDs, and their offspring, are characterized by being healthier than age-matched controls born from non-long-living parents, i.e. a slower aging and are biologically younger than their chronological age of about 9 and 5 years, respectively. Particularly important within the scenario of COVID-19 pandemic is that centenarians have a peculiar state/degree of inflammaging, which is much lower than that predicted by their chronological age and is biased toward anti-inflammaging, i.e. the production of anti-inflammatory molecules and cells that the body produces lifelong as an adaptive, compensatory mechanisms to continuously down-regulate the inflammatory process and avoid its chronic detrimental effects [18–20]. Accordingly, the oldest old, including centenarians, are high-selected, exceptionally robust subjects that can be taken as a model of successful/healthy aging [21].

A major characteristic of human longevity is the ubiquitous female survival advantage. In particular, centenarian females outnumber males [22], and this demographic phenomenon is particularly evident in Northern Italy, including Lombardy [23]. However, although women live longer, they suffer greater morbidity, particularly late in life. In Trieste, a city situated in the North-East of Italy with 204,000 inhabitants, the prevalence of centenarians is high: in mid-June 2020 there were 148 centenarians, number obtained from the list of the public health service considering only subjects who have reached 100 years of age. In 2014 we started the Centenari a Trieste (CaT) Study, to examine the centenarians living in Trieste. From 2014 to January 2020 we enrolled, visited and collected data of 130 centenarians, using the annual lists provided by the public health service mentioned above. 90% of our centenarian population are women, but the few males are all in excellent health [24]. The complex reasons of such a female longevity advantage/paradox is still unclear [25, 26] but it is likely the result of a mixture of biological (e.g. genetics) and non-biological (e.g. cultural, anthropological) factors [27, 28].

Within this scenario, and considering that the population age 100 years and older is part of the fastest growing segment of the population worldwide, we thought worthwhile to check COVID-19-related vulnerability/mortality in old people across the above-mentioned large and heterogeneous age spectrum, focusing on nonagenarians and centenarians and gender, in Lombardy, the largest (10 million inhabitants) and most populous Italian Region, heavily affected by COVID-19 pandemic. To this regard, our study refers to the peak of infection (March 2020) when the number of (reported) infected people was 76,586 and the number of deaths was 11,399 (data from the Italian Civil Protection Department available at: http://opendatadpc.maps.arcgis.com/apps/opsdashboard/index.html-/b0c68bce2cce478eaac82fe38d4138b1).

The following questions/ hypotheses were addressed/ tested: i) is the COVID-19-related mortality of exceptionally long-living subjects, lower than that of people in the age-range between 50 and 80 years of age? ii) do the COVID-19-related mortality data show any gender difference in nonagenarians and centenarians?

RESULTS

Lombardy municipalities

During March 2020 a large increase in mortality was seen in Lombardy relative to previous years, both in absolute and in relative terms: against a background of 8492 deaths (mean of March deaths between 2015 and 2019), in 2020 there were 24,330 deaths, constituting an increase of 15,838 in absolute numbers and of 286% in percentage. Men contributed more to this increase with 9021 (57.0%) extra deaths. Increase in mortality is apparent in older age groups. In fact, while excess mortality under 40 years of age totalized less than 50 persons, its maximum was reached in the 80-84 and 85-89 age categories, with about 3300 more deaths each (Supplementary Figure 1).

When percent excess death by age class was plotted, a continuous increase in mortality by age was apparent (Figure 1, panel A). This phenomenon was clearly visible in both men (Figure 1, panel B) and in women (Figure 1, panel C), where March 2020 mortality is compared with mean March mortality of the previous years. However, the two patterns had also some differences: in women the increase resulted in approximately a doubling in mortality risk in each age class, whereas in men the greatest relative increase was in "younger" ages, were 2020 mortality was more than three times that of previous years, while in later ages the increase is about 80%. These different changes result in two different patterns of increase by age, i.e. women increase in excess mortality was lower in "younger"

ages, but reached that of men in later ages (Figure 1, panel D): while excess mortality under 90 was much higher in men, it was similar in the nonagenarians and in centenarians women even had a higher mortality.

We tested if the increase in mortality by age was different between men and women entering an interaction (age*sex) term in a logistic model: the effect was statistically significant ($p<0.0001$). We further refined the model entering age also as a quadratic term, together with its interaction with sex (age squared*sex): the interaction term resulted statistically significant ($p<0.0001$). This latter model was statistically better than the simpler one ($p<0.0001$). Both models indicated that the probability of dying was much higher in "young" men, but that at older ages the difference was less pronounced (simpler model) or even reversed (model with quadratic age).

Mortality in Trieste

Owing to the above-mentioned large heterogeneity of the health status of elderly subjects including nonagenarians and centenarians [12–14], and considering that morbidity, mortality and longevity outcomes are largely context-dependent [27, 28], it is interesting to look at what can be observed with a higher "granularity".

In March 2020 in Trieste there were 138 centenarians, 90% of them were women (Figure 2). 71 centenarians were tested with swab for COVID-19: three of them resulted positive but subsequently became negative at test and were therefore considered cured of COVID-19 infection. The remaining 68 centenarians tested negative for COVID-19: four of them died of old age from March to mid-June 2020.

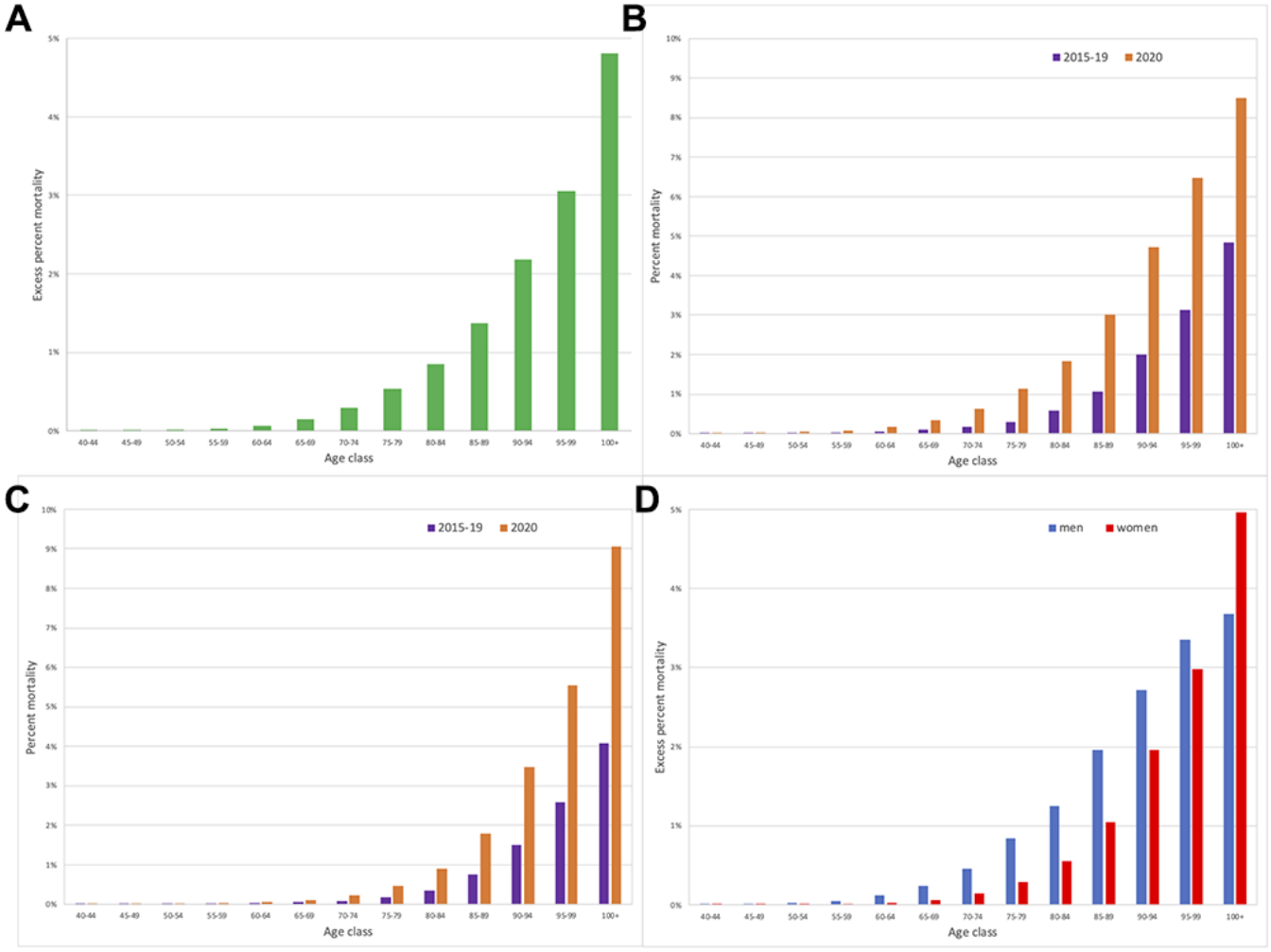

Figure 1. Mortality in March 2020 in Lombardy compared with mean mortality in March in 2015-2019. (**A**) Percent March 2020 excess mortality, by age class. (**B**) Men percent mortality by age class and year. (**C**) Women percent mortality by age class and year. (**D**) Percent March 2020 excess mortality by age class and sex.

As part of the CaT Study, from October 2019 to January 2020, immediately before medical emergency for COVID-19, we enrolled 42 centenarians using the list of the public health service, 39 women and 3 men (Figure 2). Six centenarians died before April 2020: five women for senectus and without symptoms related to COVID-19 infection. A man was admitted in a ward COVID-19 infected and was the only one dying with COVID-19 pathology. A woman of the 42 centenarians enrolled in the Study tested for COVID-19 was negative despite living in a nursing home with a COVID-19 outbreak, where most of the other elderly guests became positive. She was one of the centenarians belonging to the group of 68 tested negative and mentioned above.

DISCUSSION

The answer to the first question (is the COVID-19-related mortality of exceptionally long-living subjects lower than that of people in the age-range between 50 and 80 years of age?) is negative. In a region such as Lombardy which experienced a high SARS-CoV-2 infection rate, we found a continuous increase in mortality by age when percent excess death by age class was plotted. On the whole, nonagenarians and centenarians, despite their capability to survive until an extreme age and to avoid/postpone most of the ARDs, could be highly vulnerable during personal and societal stressful events like as seen during the SARS-CoV-2 pandemic. These data are in accord with the hypothesis that a major reason of such increasing vulnerability of the elderly, including nonagenarians and centenarians, to COVID-19 infection and related stressful conditions is inflammaging, the age-related increase of the inflammatory status which is particularly deleterious in those old subjects affected by one or more comorbidities. Inflammaging is a complex phenomenon at present only partially understood which can be highly different and personalized in different individuals [29]. Accordingly, the conceptual framework of inflammaging could help in understanding both the higher vulnerability of the elderly to COVID-19 but also the different responsiveness to COVID-19 infection and related contextual stressors in different subsets of elderly people.

The take home message is that nonagenarians and centenarians need particular attention, protection and special care in situations challenging the capability of hospitals, nursing homes and Health Service to cope with exceptional events like the COVID-19 pandemic.

However, when mortality is disentangled according to gender a peculiar gender-specific crossing emerged. The excess of mortality presumably due, directly or indirectly, to COVID-19 explosively grew in males from 50 years of age up to 80 years but thereafter the

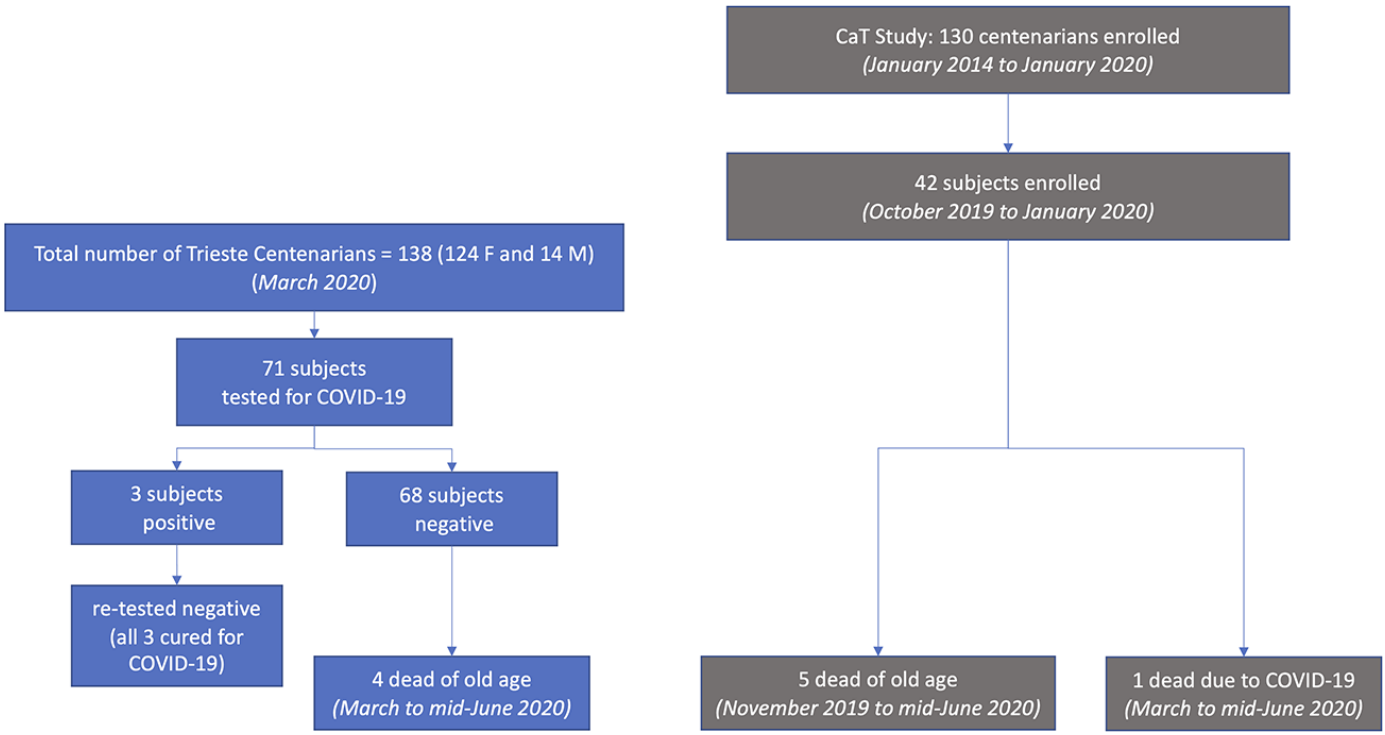

Figure 2. COVID-19 testing and deaths in Trieste (left) and in the CaT (Centenari a Trieste) Study (right).

rise tended to slow down. Females had a similar age trend, but their risk was lower in lower ages than in males and the decrease in higher age groups was less marked. Thus, very old people such as nonagenarian and centenarian males appears to be more resilient than age-matched females.

Accordingly, the answer to the second question (do the COVID-19-related mortality data show any gender difference in nonagenarians and centenarians?) is positive.

The reasons of such gender-specific trajectories of resilience are unclear. We reported that in men a genetic predisposition to produce high levels of IL-6 is detrimental for longevity [30]. In subjects with ages ranging from 22 to 93 years the age-related decline in adaptive immunity (particularly T cells) and especially the activation of innate immunity despite being present in both women and men were significantly greater in magnitude in men, suggesting that they experience a stronger inflammaging than women even when the subjects were otherwise healthy and clinically comparable in terms of age, BMI, and ethnicity [31]. Men have also a stronger inflammatory state in circulating monocytes compared to women [31]. Thus, men-specific immune characteristics interacting with/related to inflammaging, such as a blunted acquired immune system and type I interferon response, coupled with the downregulation of ACE2 (SARS-CoV-2 receptor) (particularly in patients with age-related comorbid diseases such as type II diabetes) and an accelerated biological aging (measured by epigenetic markers and telomere shortening), could help in explaining the higher vulnerability of men to COVID-19 infection [32].

To understand why men older than about 90 years become relatively more resilient than age-matched females it is important to consider the above-mentioned female-male health-survival paradox [25, 26, 33]. Indeed, despite women live longer than men and appear to be stronger even during severe famines and epidemics [34] when they became nonagenarians and centenarians show a much worse health status than that of nonagenarian and centenarian men who have a much better physical and cognitive health. The more years of life expectancy of women are mostly years of disease and disability [27, 28]. In any case, nonagenarians and centenarians are a mix of those aging well and those aging poorly, and in this heterogeneous scenario men capable of reaching age 90 and especially 100 are likely the more robust. Centenarian men are fewer but more selected and healthier and likely more resilient than centenarian women in highly stressful conditions like COVID-19 pandemic.

Finally, it is important to note that the two general conclusions of our study, i.e. the high COVID-19-related mortality of nonagenarians and centenarians and the relative resilience of male centenarians, resulting from epidemiological investigations can be at variance with anecdotal observations that centenarians and sometime supercentenarians (people over 110 years old) survived and recovered after COVID-19 infection. As an example, our data on a low number of very well characterized subjects of the CaT Study, suggest that both centenarian women and men looked strong during the peak of COVID-19 pandemic which profoundly challenged the entire health system and care of the elderly. What can be observed and reported at a higher magnification and higher granularity in single cities, institutions and settings is the consequence of the basic heterogeneity of the aging phenotype which is particularly evident at the extreme ages and suggests that outcomes may differ by robustness or other characteristics of the individual and are always highly diverse and context-dependent. To this regard, it can be predicted that the use of proteomic [15], epigenetic [16, 17] and glycomic biomarkers [35], among others, capable of distinguishing between chronological and biological age, will help in disentangling the heterogeneity of the aging phenotypes and in identifying the elderly characterized by an accelerated aging and lower robustness and thus at higher risk of morbidity and mortality in normal as well in exceptional circumstances such the COVID-19 pandemic.

Strength and limitations

We had access to open data provided by Istat, which despite being a non-representative subset of Italian municipalities covers the Lombardy population almost completely (about 97%). The data on the entire Italian population would have diluted the results here presented, owing to the much lower mortality in the other Italian regions.

We analyzed total mortality and not COVID-19-related deaths. This is both a limitation and a strength. Due to the great strain imposed on the Italian National Health Service, particularly in the hardest hit provinces, we cannot exclude an increase in general mortality due to a missing response to needs that would have been otherwise met. Even if this may not be excluded, we find difficult to think of logistical reasons that would differentially impact men and women and spare oldest men. Analyzing only confirmed COVID-19 deaths is more specific but, due to the impressive surge in mortality, only a part of those who died due to the infection were reported as being infected, and only a part of them was subject to a verification. Also, in absence of clear typical manifestations, a part of

COVID-19 mortality could be incorrectly attributed to other causes, even after a closer reanalysis, since swabs were only partially available, and their sensitivity is far from perfect.

In conclusion, we reported data that clearly show that old people, including nonagenarians and centenarians, suffered a high COVID-19-related mortality in the Lombardy region and suggested that the conceptual framework of inflammaging could help in understanding such age-related vulnerability. The remarkable difference between women and men in life expectancy, disability, mortality and longevity which emerged also in circumstances such as the COVID-19 pandemic is complex but still poorly understood and deserves attention and a closer scrutiny. Preventive strategies focused on the elderly preparing us better for the next pandemic are urgently needed [6].

MATERIALS AND METHODS

We used publicly available online data from the Istat (Italian Institute of Statistics) site: https://www.istat.it/it/archivio/240401 (accessed on June 15, 2020). Mortality raw data in a large dataset of Italian municipalities were collected by ANPR (National Registry of Resident Population) operated by the Ministry of the Interior. These data were successively merged with the dataset of the Registry Tax operated by the Ministry of Economy and Finance, validated and made available on-line by Istat.

Mortality data were made available for each day starting from January 1 to Apr 30, 2020 by municipality, 5-year age classes, and sex. Reference mortality data are available for the years 2015 to 2019, with same granularity.

Since we wanted to study the effect of the virus on mortality by age we concentrated on the Lombardy region, which presently (June 15, 2020) accounts for almost half of the confirmed COVID-19 deaths in Italy, and on the peak of infection (March 2020). Notably the dataset covers 97.1% of the Lombardy population.

Population in Italy (and as a consequence also in Lombardy) is gradually ageing, rendering impossible a direct comparison of 2020 deaths to 2015-2019 deaths. In order to correct for this imbalance we calculated the 2020 death percentage comparing the number of deaths within age classes with the respective age class populations, i.e. [(March 2020 number of deaths)/(March 2020 Lombardy population)]*100, for each age class, by sex. Reference 2015-2019 death percentage was calculated similarly as [(mean March 2015-2019 number of deaths)/(mean March 2015-2019 Lombardy population)] *100, for each age class, by sex. Percent excess mortality was calculated as a difference between 2020 mortality percentage and previous years mean mortality percentage. Lombardy population data was retrieved from the demo.istat.it site. Population data for 2020 is not available yet, so we used the data from the Istat population projections for 2020 available from same site.

We used logistic regression models in which age and sex were used as predictors for March 2020 probability of excess mortality, i.e. we disregarded "usual" (mean 2015-2019) number of March deaths. Age was modelled as a continuous factor, and age classes were given an intermediate value: for example 80-84 class was given an 82.5 value. Last class (100+) was given a 102.5 value. Models tried were hierarchically related: first only age and sex, then an interaction age*sex was entered into the model to test if the rate of increase was different between sexes. A quadratic effect, and its interaction was tried in a subsequent model.

The protocol of the CaT study to obtain, after informed consent, demographic, anamnestic, clinical and lifestyle data from the subjects enrolled in the study was already published [36].

JMP Pro v 15.0 (SAS Institute Inc.) was used to manage data and perform statistics.

Abbreviations

ACE: Angiotensin-converting-enzyme; ARD: age-related diseases; BMI: body mass index; CaT: Centenari a Trieste; COVID-19: coronavirus disease 2019; SARS-CoV-2: severe acute respiratory syndrome coronavirus; IL: interleukin.

AUTHOR CONTRIBUTIONS

GM, GiuF, and MS collected data; MT performed data analysis; GM, CF and MT prepared draft manuscript; AN, CF, GiaF and GC critically reviewed manuscript; all the authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

No financial or conflicts of interest related to the manuscript.

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REFERENCES

1. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. Science. 2020; 368:473–74. https://doi.org/10.1126/science.abb8925 PMID:32303591

2. Messner CB, Demichev V, Wendisch D, Michalick L, White M, Freiwald A, Textoris-Taube K, Vernardis SI, Egger AS, Kreidl M, Ludwig D, Kilian C, Agostini F, et al. Ultra-high-throughput clinical proteomics reveals classifiers of COVID-19 infection. Cell Syst. 2020; 11:11–24.e4. https://doi.org/10.1016/j.cels.2020.05.012 PMID:32619549

3. Remuzzi A, Remuzzi G. COVID-19 and Italy: what next? Lancet. 2020; 395:1225–28. https://doi.org/10.1016/S0140-6736(20)30627-9 PMID:32178769

4. Mueller AL, McNamara MS, Sinclair DA. Why does COVID-19 disproportionately affect older people? Aging (Albany NY). 2020; 12:9959–9981. https://doi.org/10.18632/aging.103344 PMID:32470948

5. Storci G, Bonifazi F, Garagnani P, Olivieri F, Bonafe M. How studies on inflamm-aging may help to understand and combat COVID-19 pandemic. PrePrints. 2020; 2020040158. [Epub ahead of print]. https://doi.org/10.20944/preprints202004.0158.v1

6. Fuellen G, Liesenfeld O, Kowald A, Barrantes I, Bastian M, Simm A, Jansen L, Tietz-Latza A, Quandt D, Franceschi C, Walter M. The preventive strategy for pandemics in the elderly is to collect in advance samples & data to counteract chronic inflammation (inflammaging). Ageing Res Rev. 2020; 62:101091. https://doi.org/10.1016/j.arr.2020.101091 PMID:32454090

7. Márquez EJ, Trowbridge J, Kuchel GA, Banchereau J, Ucar D. The lethal sex gap: COVID-19. Immun Ageing. 2020; 17:13. https://doi.org/10.1186/s12979-020-00183-z PMID:32457811

8. Kennedy BK, Berger SL, Brunet A, Campisi J, Cuervo AM, Epel ES, Franceschi C, Lithgow GJ, Morimoto RI, Pessin JE, Rando TA, Richardson A, Schadt EE, et al. Geroscience: linking aging to chronic disease. Cell. 2014; 159:709–13. https://doi.org/10.1016/j.cell.2014.10.039 PMID:25417146

9. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014 (Suppl 1); 69:S4–9. https://doi.org/10.1093/gerona/glu057 PMID:24833586

10. Franceschi C, Garagnani P, Morsiani C, Conte M, Santoro A, Grignolio A, Monti D, Capri M, Salvioli S. The continuum of aging and age-related diseases: common mechanisms but different rates. Front Med (Lausanne). 2018; 5:61. https://doi.org/10.3389/fmed.2018.00061 PMID:29662881

11. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, Ferrucci L, Gilroy DW, Fasano A, Miller GW, Miller AH, Mantovani A, Weyand CM, et al. Chronic inflammation in the etiology of disease across the life span. Nat Med. 2019; 25:1822–32. https://doi.org/10.1038/s41591-019-0675-0 PMID:31806905

12. Franceschi C, Salvioli S, Garagnani P, de Eguileor M, Monti D, Capri M. Immunobiography and the heterogeneity of immune responses in the elderly: a focus on inflammaging and trained immunity. Front Immunol. 2017; 8:982. https://doi.org/10.3389/fimmu.2017.00982 PMID:28861086

13. Ciabattini A, Nardini C, Santoro F, Garagnani P, Franceschi C, Medaglini D. Vaccination in the elderly: the challenge of immune changes with aging. Semin Immunol. 2018; 40:83–94. https://doi.org/10.1016/j.smim.2018.10.010 PMID:30501873

14. Conte M, Ostan R, Fabbri C, Santoro A, Guidarelli G, Vitale G, Mari D, Sevini F, Capri M, Sandri M, Monti D, Franceschi C, Salvioli S. Human aging and longevity are characterized by high levels of mitokines. J Gerontol A Biol Sci Med Sci. 2019; 74:600–07. https://doi.org/10.1093/gerona/gly153 PMID:29955888

15. Lehallier B, Gate D, Schaum N, Nanasi T, Lee SE, Yousef H, Moran Losada P, Berdnik D, Keller A, Verghese J, Sathyan S, Franceschi C, Milman S, et al. Undulating changes in human plasma proteome profiles across the lifespan. Nat Med. 2019; 25:1843–50. https://doi.org/10.1038/s41591-019-0673-2 PMID:31806903

16. Horvath S, Pirazzini C, Bacalini MG, Gentilini D, Di Blasio AM, Delledonne M, Mari D, Arosio B, Monti D, Passarino G, De Rango F, D'Aquila P, Giuliani C, et al. Decreased epigenetic age of PBMCs from italian semi-supercentenarians and their offspring. Aging (Albany NY). 2015; 7:1159–70.

https://doi.org/10.18632/aging.100861
PMID:26678252

17. Franceschi C, Ostan R, Santoro A. Nutrition and inflammation: are centenarians similar to individuals on calorie-restricted diets? Annu Rev Nutr. 2018; 38:329–56. https://doi.org/10.1146/annurev-nutr-082117-051637 PMID:29852087

18. Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S. Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev. 2007; 128:92–105. https://doi.org/10.1016/j.mad.2006.11.016 PMID:17116321

19. Sayed N, Gao T, Tibshirani R, Hastie T, Cui L, Kuznetsova T, Rosenberg-Hasson Y, Ostan R, Monti D, Lehallier B, Shen-Orr S, Maecker HT, Dekker CL, et al. An Inflammatory Clock Predicts Multi-morbidity, Immunosenescence and Cardiovascular Aging. Humans BioRxiv. 2019. https://doi.org/10.1101/840363

20. Storci G, De Carolis S, Papi A, Bacalini MG, Gensous N, Marasco E, Tesei A, Fabbri F, Arienti C, Zanoni M, Sarnelli A, Santi S, Olivieri F, et al. Genomic stability, anti-inflammatory phenotype, and up-regulation of the RNAseH2 in cells from centenarians. Cell Death Differ. 2019; 26:1845–58. https://doi.org/10.1038/s41418-018-0255-8 PMID:30622304

21. Franceschi C, Bonafè M. Centenarians as a model for healthy aging. Biochem Soc Trans. 2003; 31:457–61. https://doi.org/10.1042/bst0310457 PMID:12653662

22. Passarino G, Calignano C, Vallone A, Franceschi C, Jeune B, Robine JM, Yashin AI, Cavalli Sforza LL, De Benedictis G. Male/female ratio in centenarians: a possible role played by population genetic structure. Exp Gerontol. 2002; 37:1283–89. https://doi.org/10.1016/s0531-5565(02)00140-7 PMID:12470842

23. Montesanto A, De Rango F, Pirazzini C, Guidarelli G, Domma F, Franceschi C, Passarino G. Demographic, genetic and phenotypic characteristics of centenarians in Italy: focus on gender differences. Mech Ageing Dev. 2017; 165:68–74. https://doi.org/10.1016/j.mad.2017.04.008 PMID:28461103

24. Brodaty H, Woolf C, Andersen S, Barzilai N, Brayne C, Cheung KS, Corrada MM, Crawford JD, Daly C, Gondo Y, Hagberg B, Hirose N, Holstege H, et al. ICC-dementia (international centenarian consortium - dementia): an international consortium to determine the prevalence and incidence of dementia in centenarians across diverse ethnoracial and sociocultural groups. BMC Neurol. 2016; 16:52. https://doi.org/10.1186/s12883-016-0569-4 PMID:27098177

25. Oksuzyan A, Juel K, Vaupel JW, Christensen K. Men: good health and high mortality. Sex differences in health and aging. Aging Clin Exp Res. 2008; 20:91–102. https://doi.org/10.1007/BF03324754 PMID:18431075

26. Austad SN, Fischer KE. Sex differences in lifespan. Cell Metab. 2016; 23:1022–33. https://doi.org/10.1016/j.cmet.2016.05.019 PMID:27304504

27. Giuliani C, Garagnani P, Franceschi C. Genetics of human longevity within an eco-evolutionary nature-nurture framework. Circ Res. 2018; 123:745–72. https://doi.org/10.1161/CIRCRESAHA.118.312562 PMID:30355083

28. Franceschi C, Garagnani P, Olivieri F, Salvioli S, Giuliani C. The contextualized genetics of human longevity: JACC focus seminar. J Am Coll Cardiol. 2020; 75:968–79. https://doi.org/10.1016/j.jacc.2019.12.032 PMID:32130932

29. Franceschi C, Zaikin A, Gordleeva S, Ivanchenko M, Bonifazi F, Storci G, Bonafè M. Inflammaging 2018: an update and a model. Semin Immunol. 2018; 40:1–5. https://doi.org/10.1016/j.smim.2018.10.008 PMID:30392751

30. Bonafè M, Olivieri F, Cavallone L, Giovagnetti S, Mayegiani F, Cardelli M, Pieri C, Marra M, Antonicelli R, Lisa R, Rizzo MR, Paolisso G, Monti D, Franceschi C. A gender—dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. Eur J Immunol. 2001; 31:2357–61. https://doi.org/10.1002/1521-4141(200108)31:8<2357::aid-immu2357>3.0.co;2-x PMID:11500818

31. Márquez EJ, Chung CH, Marches R, Rossi RJ, Nehar-Belaid D, Eroglu A, Mellert DJ, Kuchel GA, Banchereau J, Ucar D. Sexual-dimorphism in human immune system aging. Nat Commun. 2020; 11:751. https://doi.org/10.1038/s41467-020-14396-9 PMID:32029736

32. Bonafè M, Prattichizzo F, Giuliani A, Storci G, Sabbatinelli J, Olivieri F. Inflamm-aging: why older men are the most susceptible to SARS-CoV-2 complicated outcomes. Cytokine Growth Factor Rev. 2020; 53:33–37. https://doi.org/10.1016/j.cytogfr.2020.04.005 PMID:32389499

33. Hoogendijk EO, van der Noordt M, Onwuteaka-Philipsen BD, Deeg DJ, Huisman M, Enroth L, Jylhä M. Sex differences in healthy life expectancy among nonagenarians: a multistate survival model using data from the vitality 90+ study. Exp Gerontol. 2019; 116:80–85. https://doi.org/10.1016/j.exger.2018.12.015 PMID:30590122

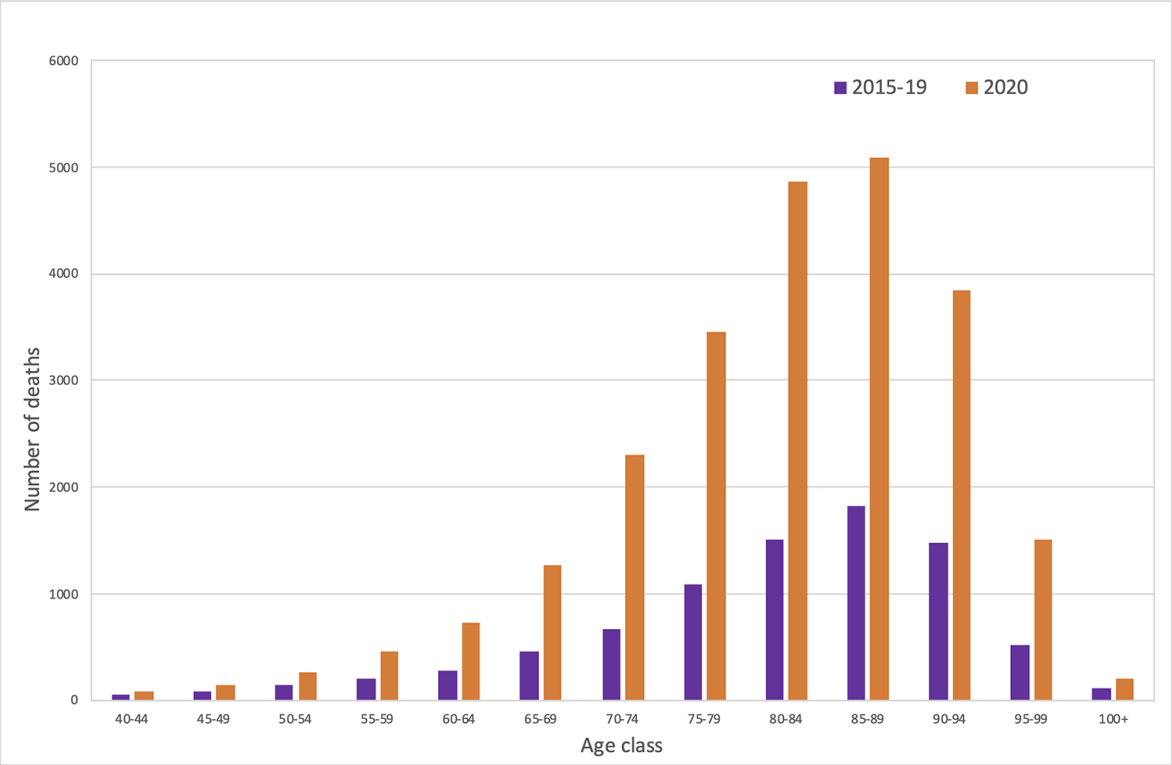
34. Zarulli V, Barthold Jones JA, Oksuzyan A, Lindahl-Jacobsen R, Christensen K, Vaupel JW. Women live longer than men even during severe famines and epidemics. Proc Natl Acad Sci USA. 2018; 115:E832–40. https://doi.org/10.1073/pnas.1701535115 PMID:29311321

35. Dall'Olio F, Vanhooren V, Chen CC, Slagboom PE, Wuhrer M, Franceschi C. N-glycomic biomarkers of biological aging and longevity: a link with inflammaging. Ageing Res Rev. 2013; 12:685–98. https://doi.org/10.1016/j.arr.2012.02.002 PMID:22353383

36. Tettamanti M, Marcon G. Cohort profile: 'Centenari a trieste' (CaT), a study of the health status of centenarians in a small defined area of Italy. BMJ Open. 2018; 8:e019250. https://doi.org/10.1136/bmjopen-2017-019250 PMID:29439076

SUPPLEMENTARY MATERIAL

Supplementary Figure



Supplementary Figure 1. Total number of deaths in March in Lombardy, by age class and year.

Research Paper

Elevated Lactate Dehydrogenase (LDH) level as an independent risk factor for the severity and mortality of COVID-19

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ABSTRACT

Early identification of severe patients with coronavirus disease 2019 (COVID-19) is very important for individual treatment. We included 203 patients with COVID-19 by propensity score matching in this retrospective, case-control study. The effects of serum lactate dehydrogenase (LDH) at admission on patients with COVID-19 were evaluated. We found that serum LDH levels had a 58.7% sensitivity and 82.0% specificity, based on a best cut-off of 277.00 U/L, for predicting severe COVID-19. And a cut-off of 359.50 U/L of the serum LDH levels resulted in a 93.8% sensitivity, 88.2% specificity for predicting death of COVID-19. Additionally, logistic regression analysis and Cox proportional hazards model respectively indicated that elevated LDH level was an independent risk factor for the severity (HR: 2.73, 95% CI: 1.25-5.97; P=0.012) and mortality (HR: 40.50, 95% CI: 3.65-449.28; P=0.003) of COVID-19. Therefore, elevated LDH level at admission is an independent risk factor for the severity and mortality of COVID-19. LDH can assist in the early evaluating of COVID-19. Clinicians should pay attention to the serum LDH level at admission for patients with COVID-19.

INTRODUCTION

Since the end of 2019, Wuhan, China, has experienced an outbreak of coronavirus disease 2019 (COVID-19) caused by a novel coronavirus later named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. SARS-CoV-2 is an RNA virus that can be transmitted from person to person, and all people are susceptible to

this infection. At present, COVID-19 has progressed into a pandemic and become a major global health concern. It is reported that most cases are nonsevere type with a good prognosis; however, severe cases may deteriorate rapidly to multiple organ damage, impaired immune function and even death [2]. Therefore, early identification of severe COVID-19 is very important for individual or precise management, including antiviral, organ support and intensive care unit (ICU) care, to improve the prognosis.

Lactate dehydrogenase (LDH) is an intracellular enzyme involved in anaerobic glycolysis that catalyzes the oxidation of pyruvate to lactate [3]. Serum LDH is routinely tested in various diseases clinically. It has been reported that elevated serum LDH levels are associated with poor prognosis in various diseases, especially in tumors and inflammation [4–6]. To date, studies have shown that patients with severe COVID-19 have elevated serum LDH levels [7, 8], but no study has specifically evaluated its effect on the severity and mortality of COVID-19. Therefore, this multicenter retrospective, case-control study aimed to explore whether the serum LDH levels at admission can assist in evaluating the severity and mortality of COVID-19.

RESULTS

Results of propensity score matching and baseline of patients

Sex, age, hypoproteinemia or anemia, tumor history, chronic kidney disease, stroke history, hyperlipidemia, hypertension, diabetes, coronary heart disease, viral hepatitis, smoking and drinking were included as covariates in the logistic regression model of the propensity score matching. We matched 203 patients (128 nonsevere and 75 severe cases) from among 523 patients (424 nonsevere and 99 severe cases) with laboratory confirmed SARS-Cov-2 infection by propensity score matching. The quality assessment of the propensity score matching is shown in Supplementary Figure 1, and the comparison before and after propensity score matching is shown in Table 1. Overall, the results of propensity score matching were satisfactory. After propensity score matching, the difference in covariables between the nonsevere group and the severe group were controlled within no statistical differences (Table 1).

In the current study, 26 (5.0%) out of 523 patients before propensity score matching and 16 (7.9%) out of 203 patients after propensity score matching died of COVID-19. Considering that the patients were not continuously enrolled, we cannot calculate the case fatality rate.

Comparison of laboratory indicators between the nonsevere group and the severe group

We analyzed the levels of laboratory indicators at admission between nonsevere group and severe group. There were significant differences ($P<0.05$) in the levels of white blood cells (WBCs), neutrophils, lymphocytes, C-reactive protein (CRP), fibrinogen, D-dimer, creatine kinase and LDH between two groups (Figure 1 and Supplementary Table 1). Considering the relationship among laboratory indicators, we conducted Pearson correlation analysis on these laboratory indicators with significant differences. As a result, CRP and LDH exhibited powerful correlations with other indexes (Supplementary Table 2), which suggested that CRP and LDH were significant factors associated with the severity of COVID-19.

Role of the serum LDH in severity and death among COVID-19 cases

We performed ROC curves on the above laboratory indicators with significant differences to assess their value in patients with COVID-19. Lymphocyte counts were the most specific predictor (specificity 94.7%) for severe COVID-19, but with a low sensitivity of 20.3% (Table 2). In contrast, D-dimer had a high sensitivity (86.7%) but a very poor specificity (37.5%) in predicting severe COVID-19. Overall, serum LDH levels had an AUC of 0.76 (95% CI: 0.70 - 0.83) for predicting severe COVID-19, with a 58.7% sensitivity and 82.0% specificity, based on a best cut-off of 277.00 (U/L) (Table 2). However, there seems to be no significant difference between CRP and LDH in predicting severe COVID-19 (Figure 2).

The AUC values of the above indicators, even the CRP and LDH, were not very satisfactory. Therefore, we further analyzed the role of these indicators in predicting the mortality due to COVID-19. Unexpectedly, a cut-off of 91.39 mg/L for serum CRP levels had a sensitivity of 81.3% and a specificity of 88.2% for predicting death in patients with COVID-19 (Table 2). In addition, when the best cut-off of was 359.50 U/L, serum LDH levels had an AUC of 0.92 (95% CI: 0.84 - 0.99) for predicting death due to COVID-19, with a sensitivity of 93.8% and specificity of 88.2% (Table 2). Similarly, there was no significant difference in the ROC curve between CRP and LDH (Figure 3).

Elevated serum LDH as an independent risk factor for the severity of COVID-19

We detected the risk factors for the severity of COVID-19 by univariate and multivariate logistic regression

Table 1. Baseline of included patients.

	Before matching			After matching		
	Nonsevere (n=424)	Severe (n=99)	P values	Nonsevere (n=128)	Severe (n=75)	P values
Female	209(49.3%)	39(39.4%)	0.096	52(40.6%)	31(41.3%)	1.000
Age	51.45±15.08	61.54±13.36	<0.001	57.13±14.55	58.49±13.35	0.508
Hypoproteinemia or anemia	24(5.7%)	25(25.3%)	<0.001	13(10.2%)	13(17.3%)	0.208
Tumor history	8(1.9%)	1(1.0%)	0.861	2(1.6%)	1(1.3%)	1.000
Chronic kidney disease	10(2.4%)	7(7.1%)	0.039	6(4.7%)	3(4.0%)	1.000
Stroke history	8(1.9%)	11(11.1%)	<0.001	3(2.3%)	1(1.3%)	1.000
Hyperlipidemia	48(11.3%)	8(8.1%)	0.448	11(8.6%)	7(9.3%)	1.000
Hypertension	82(19.3%)	43(43.4%)	<0.001	44(34.4%)	25(33.3%)	1.000
Diabetes	61(14.4%)	23(23.2%)	0.045	32(25.0%)	18(24.0%)	1.000
Coronary heart disease	17(4.0%)	12(12.1%)	0.003	10(7.8%)	6(8.0%)	1.000
Viral hepatitis	7(1.7%)	1(1.0%)	0.99	3(2.3%)	1(1.3%)	1.000
Smoking	27(6.4%)	13(13.1%)	0.008	10(7.8%)	9(12.0%)	0.566
Drinking	28(6.6%)	16(16.2%)	0.002	12(9.4%)	10(13.3%)	0.628
Death	0	26(26.3%)	<0.001	0	16(21.1%)	<0.001

analysis. Neutrophils were excluded from logical regression analysis because neutrophils and leukocytes were collinear. In univariate analysis, high levels of WBC (HR: 2.32, 95% CI: 1.29, 4.16; P=0.005), CRP (HR: 4.91, 95% CI: 2.61-9.24; P<0.001), neutrophil-to-lymphocyte ratio (HR: 3.51, 95% CI: 1.93-6.39; P<0.001), fibrinogen, D-dimer (HR: 3.26, 95% CI: 1.60-6.64; P=0.001), creatine kinase and LDH (HR: 6.48, 95% CI: 3.40-12.34; P<0.001), and low levels of lymphocytes (HR: 4.53, 95% CI: 1.51-13.53; P=0.007) were risk factors for the severity of COVID-19 (Table 3). Furthermore, we took indicators that were P<0.1 in univariate logistic regression into multivariate logistic regression analysis. Of the 8 indicators, the P value of

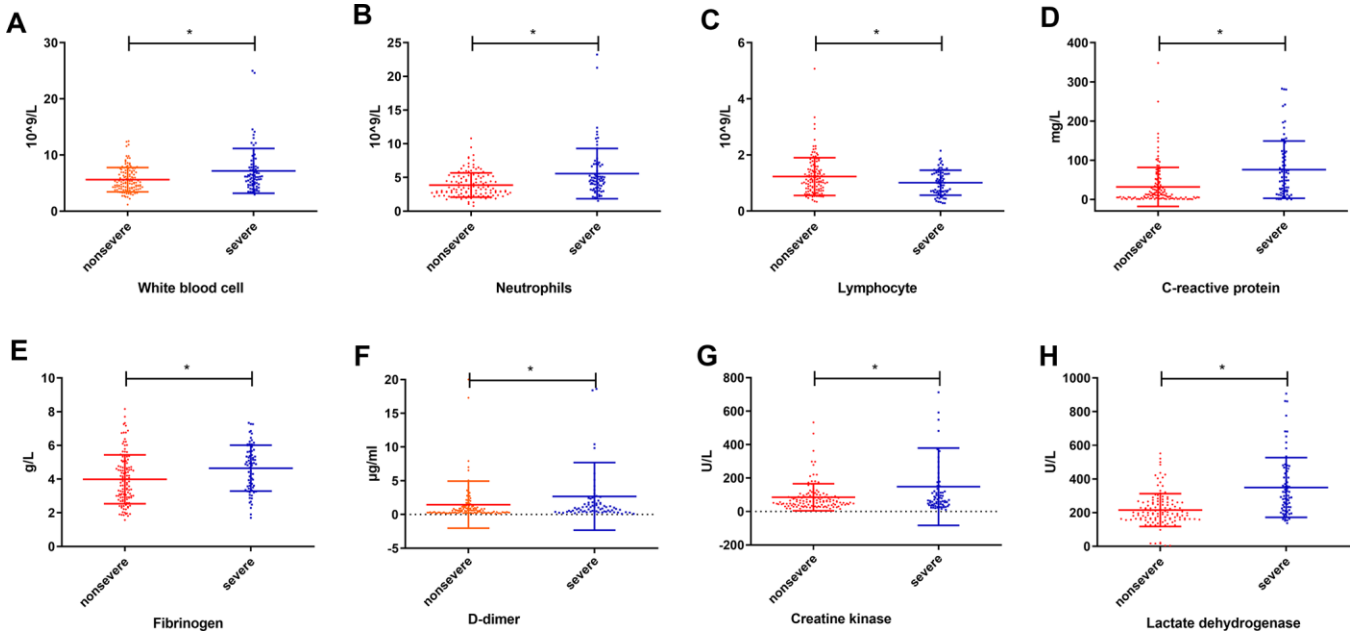

Figure 1. Levels (mean ± SD) of laboratory indicators at admission between the nonsevere group and severe group. (**A**) white blood cell; (**B**) neutrophils; (**C**) lymphocyte; (**D**) c-reactive protein; (**E**) fibrinogen; (**F**) d-dimer; (**G**) creatine kinase; (**H**) lactate dehydrogenase. * P<0.05.

Table 2. Role of laboratory indicators in predicting the severity and death of COVID-19.

	Predicting severity of COVID-19				Predicting death of COVID-19			
	AUC	Best cut-off *	Sensitivity	Specificity	AUC	Best cut-off *	Sensitivity	Specificity
WBC	0.63±0.04	5.65 (×10^9/L)	0.627	0.594	0.78±0.07	7.45(×10^9/L)	0.688	0.797
Neutrophils	0.66±0.04	3.85 (×10^9/L)	0.707	0.586	0.82±0.05	4.87(×10^9/L)	0.813	0.711
Lymphocyte	0.58±0.04	1.72 (×10^9/L)	0.203	0.947	0.76±0.06	0.73(×10^9/L)	0.759	0.750
NLR	0.68±0.04	3.83	0.640	0.660	0.87±0.06	7.42	0.750	0.900
CRP	0.73±0.04	20.14 (mg/L)	0.747	0.625	0.89±0.05	91.39 (mg/L)	0.813	0.882
Fibrinogen	0.64±0.04	4.79 (g/L)	0.533	0.758	0.69±0.06	3.96 (g/L)	0.875	0.497
D-dimer	0.65±0.04	0.33 (μg/ml)	0.867	0.375	0.80±0.06	1.09 (μg/ml)	0.813	0.706
CK	0.55±0.04	109.50 (U/L)	0.347	0.812	0.62±0.08	120.50 (U/L)	0.438	0.818
LDH	0.76±0.04	277.00 (U/L)	0.587	0.820	0.92±0.05	359.50 (U/L)	0.938	0.882

* Chosen by maximizing the Youden index. Abbreviations: AUC, area under the curve; WBC, white blood cell; NLR, neutrophil-to-lymphocyte ratio; CRP, c-reactive protein; CK, creatine kinase; LDH, lactic dehydrogenase.

the serum LDH levels was still less than 0.05, which suggested that elevated serum LDH (HR: 2.73, 95% CI: 1.25-5.97; P=0.012) is an independent risk factor for the severity of COVID-19 (Table 3).

Elevated serum LDH as an independent risk factor for mortality of COVID-19

We applied the Cox proportional hazards model to evaluate the effect of LDH on the survival time of patients. In univariable Cox regression analysis, male sex (HR: 3.04, 95%: CI 0.87-10.65; P=0.083) and age older than 60 years (HR: 5.88, 95% CI: 1.33-25.90, P=0.019) had a significant effect on the survival time of patients. In addition, elevated serum WBC count (HR: 8.06, 95% CI: 2.8-23.23; P<0.001), neutrophil-to-lymphocyte ratio (HR: 21.11, 95% CI: 6.80-65.51; P<0.001), CRP (HR: 24.06, 95% CI: 6.85-84.50; P<0.001), fibrinogen, D-dimer, CK, LDH (HR: 77.20, 95% CI: 10.20-584.61; P<0.001) and reduced lymphocyte counts were risk factors of mortality (Table 4). We take indicators that were P<0.1 in univariate logistic regression into multivariate logistic regression analysis. We found that the elevated serum LDH (HR: 40.50, 95% CI: 3.35-449.28; P=0.003) remained an independent risk factor for the mortality of COVID-19 (Table 4).

DISCUSSION

In this study, we identified that elevated serum LDH level was an independent indicator for predicting severity and mortality in patients with COVID-19 for the first time. Based on ROC analysis, serum LDH

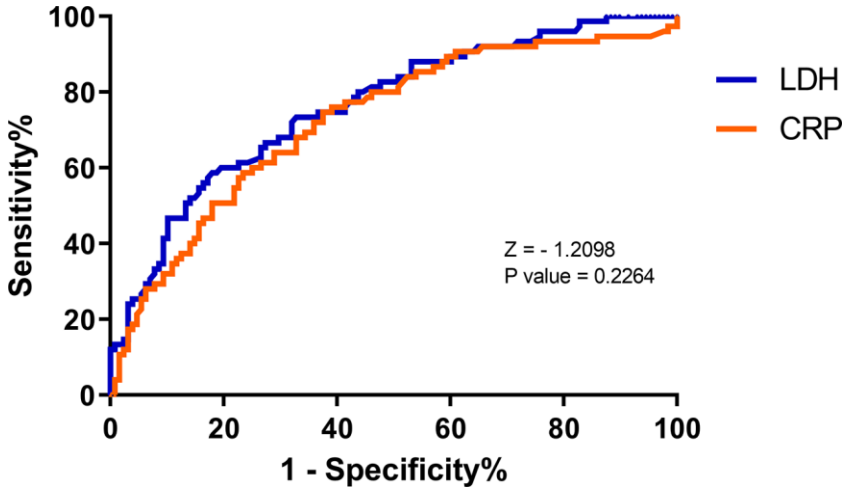

Figure 2. Receiver operating characteristic (ROC) curve for predicting severity of COVID by C-reactive protein (CRP) and lactate dehydrogenase (LDH) levels at admission. LDH: AUC 0.76 ± 0.04, cut-off 277.00 U/L, sensitivity 58.7%, specificity 82.0%. CRP: AUC 0.73 ± 0.04, cut-off 20.14 mg/L, sensitivity 74.7%, specificity 62.5%.

levels at admission had high specificity for predicting the severity of COVID-19 and a satisfactory sensitivity and specificity for predicting death due to COVID-19. Furthermore, logistic regression analysis and Cox proportional hazards model revealed that elevated serum LDH at admission to be an independent risk factor for the severity and mortality of COVID-19.

We regarded sex, age, hypoproteinemia or anemia, tumor history, chronic kidney disease, stroke history, hyperlipidemia, hypertension, diabetes, coronary heart disease, viral hepatitis, smoking and drinking as covariates in the logistic regression model of the propensity score matching, because these covariates may have an impact on the severity and mortality of COVID-19 [9–11]. Autoimmune and inflammatory diseases do have an impact on the severity and mortality of COVID-19. We did not include autoimmune and inflammatory diseases in the logistic regression model of the propensity score matching because there were no patients diagnosed with autoimmune and inflammatory diseases in the enrolled patients. After propensity score matching, the differences in covariables between the nonsevere group and the severe group were controlled at almost the same levels. Controls for confounding factors were the premise of this study, ensuring the reliability of the conclusions.

As suggested by comparison of laboratory indicators, there were significant differences in the levels of WBC, neutrophils, lymphocytes, CRP, fibrinogen, D-dimer, creatine kinase and LDH between nonsevere and severe groups. The differences in these indicators were very similar to those reported by Huang et al. [12]. Notably, LDH showed a powerful correlation with the other indexes by Pearson correlation analysis, which suggested that LDH was a significant factor associated with the severity of patients with COVID-19. When the body experiences acute hypoxia or inflammation, the level of LDH in serum will rise significantly. COVID-19, caused by SARS-Cov-2 infection, mainly involves in the lungs, as well as other tissues and organs [13, 14], leading to hypoxia, thrombogenesis, inflammation and organ injury. Theoretically, elevated serum LDH is an important laboratory indicator for evaluating COVID-19 [15].

In this study, male sex and age older than 60 years old had obvious effects on death due to COVID-19. We found that patients who were aged over 60 years (HR: 5.88, 95% CI: 1.33-25.90, P=0.019) and male (HR: 3.04, 95%: CI 0.87-10.65; P=0.083) were more likely to expire, as suggested by the univariate Cox proportional hazards model. This obtained similar general conclusions as previous studies [16, 17]. However, the effect of age and sex on death due to COVID-19 was reduced in multivariate Cox regression because the risk of age and sex was adjusted for other factors.

Elevated serum LDH as an independent risk factor for COVID-19 is the main conclusion of this study. In univariate analysis, high WBC, NLR, CRP, fibrinogen, D-dimer, creatine kinase and LDH, and low lymphocyte were not only risk factors for severity but also risk indicators for death among patients with COVID-19 (Table 3 and Table 4). Additionally, in multivariate analysis, elevated serum LDH remained an independent risk factor for COVID-19 severity and mortality. A previous study [17], which did not mention the influence of LDH on COVID-19, proved that NLR is an

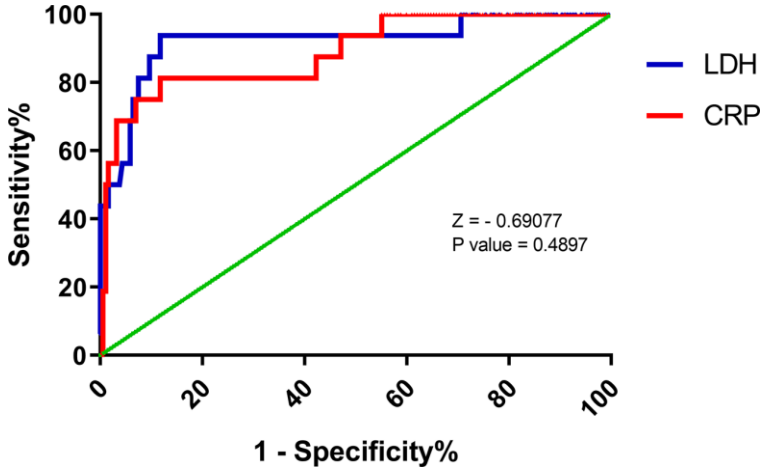

Figure 3. Receiver operating characteristic (ROC) curve for predicting death (B) of COVID by C-reactive protein (CRP) and lactate dehydrogenase (LDH) levels at admission. LDH: AUC 0.92 ± 0.05, cut-off 359.50 U/L, sensitivity 93.8%, specificity 88.2%. CRP: AUC 0.89 ± 0.05, cut-off 91.39 mg/L, sensitivity 81.3%, specificity 88.2%.

Table 3. Uni- and multivariate logistic regression analyses of risk factors for the severity of COVID-19.

Variables	Univariate logistic regression		Multivariate logistic regression	
	P value	Hazard ratio (95%CI)	P value	Hazard ratio (95%CI)
WBC* (> 5.65×10^9/L)	0.005	2.32 (1.29, 4.16)	0.056	2.01 (0.98, 4.09)
Lymphocyte* (< 1.72×10^9/L)	0.007	4.53(1.51, 13.53)	0.240	2.09 (0.61, 7.15)
NLR* (>3.83)	< 0.001	3.51 (1.93, 6.39)	0.633	1.21 (0.55, 2.64)
CRP* (> 20.14 mg/L)	< 0.001	4.91(2.61, 9.24)	0.109	1.93 (0.86, 4.31)
Fibrinogen* (> 4.79 g/L)	< 0.001	3.58(1.95, 6.57)	0.257	1.54 (0.73, 3.22)
D-dimer* (> 0.33 μg/ml)	0.001	3.26(1.60, 6.64)	0.398	1.43 (0.62, 3.29)
CK* (> 109.50 U/L)	0.012	2.30(1.20, 4.41)	0.364	1.43 (0.66, 3.08)
LDH* (> 277.00 U/L)	< 0.001	6.48(3.40, 12.34)	0.012	2.73(1.25, 5.97)

*Take the best cut-off for predicting the severity of COVID-19 as the boundary value of binary variable. Abbreviations: WBC, white blood cell; NLR, neutrophil-to-lymphocyte ratio; CRP, C-reactive protein; CK, creatine kinase; LDH, lactic dehydrogenase.

Table 4. Uni- and multivariate Cox regression analyses of risk factors for the death due to COVID-19.

Variables	Univariate Cox regression		Multivariate Cox regression	
	P value	Hazard ratio (95%CI)	P value	Hazard ratio (95%CI)
Sex (male)	0.083	3.04 (0.87, 10.65)	0.876	1.13 (0.25, 5.14)
Age (> 60)	0.019	5.88 (1.33, 25.90)	0.914	1.12 (0.15, 8.13)
WBC* (> 7.45×10^9/L)	< 0.001	8.06 (2.80, 23.23)	0.245	2.46 (0.54, 11.19)
Lymphocyte* (< 0.73×10^9/L)	< 0.001	7.47 (2.41, 23.18)	0.843	1.17 (0.24, 5.71)
NLR* (>7.42)	< 0.001	21.11 (6.80, 65.51)	0.131	4.33 (0.65, 28.95)
CRP* (> 91.39 mg/L)	< 0.001	24.06 (6.85, 84.50)	0.558	1.82 (0.25, 13.52)
Fibrinogen* (> 3.96 g/L)	0.016	6.19 (1.41, 27.21)	0.846	1.23 (0.15, 9.76)
D-dimer* (> 1.09 μg/ml)	0.001	8.67 (2.47, 30.45)	0.476	0.51 (0.08, 3.22)
CK* (> 120.50 U/L)	0.023	3.14 (1.17, 8.42)	0.827	1.13 (0.37, 3.41)
LDH* (> 359.50 U/L)	< 0.001	77.20 (10.20, 584.61)	0.003	40.50(3.65, 449.28)

*Take the best cut-off for predicting death due to COVID-19 as the boundary value of binary variable. Abbreviations: WBC, white blood cell; NLR, neutrophil-to-lymphocyte ratio; CRP, C-reactive protein; CK, creatine kinase; LDH, lactic dehydrogenase.

independent risk factor for in-hospital mortality in COVID-19. Therefore, we included the NLR in Cox proportional hazards model. However, in our study, we proved that LDH was a more independent risk factor compared with NLR as suggested by multivariate Cox regression (Table 4).

There are some limitations in this study that should be noted. Firstly, the number of subjects included is to some extent small which limits the statistical power of this study. Nonetheless, the sample size of this study was sufficient to draw our conclusion. Secondly, on a whole, 16 out of 203 patients died of COVID-19 in this study. Considering the small number of deaths, we performed Cox regression instead of logistic regression to analyze the effect of LDH on COVID-19 mortality. Although the 95% confidence interval of HR is slightly lager, it is enough to ensure that elevated serum LDH is an independent risk indicator for death due to COVID-19. Thirdly, although we have controlled the bias by propensity score matching, multiple potential confounders might not have been fully considered. A small number of patients have taken antiviral drugs, antihypertensive drugs, and antidiabetic drugs prior to admission, the effect of past medical history on the results were not studied.

In conclusion, this study revealed that serum LDH at admission was useful in evaluating the disease severity and in-hospital mortality among patients with COVID-19. Further studies are needed to confirm our findings.

MATERIALS AND METHODS

Study design and participants

We collected data for 523 adult patients admitted to the hospital with laboratory confirmed SARS-Cov-2 infection in 4 designated tertiary hospitals in Hubei

Province, including 2 in Wuhan city and 2 in cities outside Wuhan, Hubei Province, from January 22, 2020 to March 14, 2020. We divided all these 523 patients into two groups: a severe group (severe type and critical severe type of COVID-19) and a nonsevere group (mild type and moderate type of COVID-19).

Considering that this study is a retrospective study, we used propensity score matching [18] to reduce biases and confounders. Ultimately, 203 patients with COVID-19 (75 patients in the severe group and 128 patients in the nonsevere group) were included.

Inclusion and exclusion criteria

Patients who met all the following criteria were included: (1) ≥18 years old, male or female; (2) laboratory confirmed SARS-Cov-2 infection; (3) complete clinical, laboratory, imaging and outcome data. Patients younger than 18 years old, with uncomplete clinical information because of transferring to other designated hospitals were excluded.

Ethical considerations

This study was approved by the Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University (ZDWY2020-K173-1). Written informed consent was waived by the Ethics Committee in consideration of the designated hospital for emerging infectious disease.

Data collection

The data included basic clinical information, diagnosis, comorbidity, and laboratory data at admission including routine blood examination, liver and renal function, myocardial enzyme, blood coagulation, procalcitonin (PCT), CRP and LDH. Additionally, neutrophil-to-lymphocyte ratio (NLR) was calculate. All these data were obtained with a standardized data collection form created by EpiData software (version 3.1). All data were checked by two physicians (Lingling Wang and Jianfang Ye) and a third researcher (Yameng Fan) adjudicated any difference in interpretation between the two primary reviewers.

Diagnosis and classification of COVID-19

COVID-19 was diagnosed and classified according to the newest "Guidelines for the Diagnosis and Treatment of COVID-19 (Trial Version 7)" [19] by the National Health Commission in China (http://www.nhc.gov.cn/). Clinical condition classification criteria are as follows: (1) mild type - clinical symptoms were mild, and no radiological changes; (2) moderate type - fever, respiratory tract or other symptoms, and pneumonia can be seen on imaging; (3) severe type - respiratory rate ≥ 30 times per minute, or the oxygen saturation is lower than 93% at rest state, or the ratio of arterial partial pressure of oxygen (PaO_2) and fraction of inspired oxygen (FiO_2) is lower than 300 mmHg (altitude below 1000 meters), or pulmonary imaging indicate that lung damage deteriorates rapidly within 24 to 48 hours; (4) critical severe type - respiratory failure requiring mechanical ventilation, or signs indicating shock, or multiple organ failure requiring admission to the intensive care unit.

Statistical analysis

Propensity score matching was performed using open source R software (version 3.6.3, Vienna, Austria) based on the "MatchIt" package [20]. The calipers value was set to 0.03, the matching ratio was 1:2, and the matching method was "nearest". Statistical analysis was performed using IBM SPSS software (version 25.0, Chicago, USA). Statistical charts were generated using GraphPad Prism software (version 8.0, San Diego, USA). The statistical results are presented as mean ± standard deviation. Continuous data were analyzed by the Student's t-tests, and the Levene test was used to decide homogeneity of variance. The receiver operating characteristic (ROC) curve, sensitivity, specificity and area under the curve (AUC) were measured to evaluate the levels of laboratory indicators in predicting the severity and mortality of COVID-19. Differences between AUCs were detected by the Z-test. All indicators were further tested by univariate and multivariate logistic regression or Cox regression analysis. The hazard ratio (HR) and 95% confidence intervals (95% CI) are shown. P value less than 0.05 was considered statistically significant.

AUTHOR CONTRIBUTIONS

Jianfang Ye designed the idea and drafted the manuscript. Chang Li, Weihua Hu, Zaishu Chen, Wei Xiao, and Qijian Chen provided the clinical data. Jianfang Ye, Lingling Wang, Yameng Fan, Zhanjin Lu, Shiyan Chen, Wei Xiao, Zaishu Chen and Junlu Tong collected the data for this study. Jianfang Ye, Lingling Wang and Yameng Fan checked the data for this study. Jie Chen performed the statistical analysis. Jin Mei and Hongyun Lu guided the entire process of this study and checked the final manuscript.

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CONFLICTS OF INTEREST

All these authors declare no conflicts of interest.

FUNDING

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REFERENCES

1. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

2. Feng Y, Ling Y, Bai T, Xie Y, Huang J, Li J, Xiong W, Yang D, Chen R, Lu F, Lu Y, Liu X, Chen Y, et al. COVID-19 with different severities: a multicenter study of clinical features. Am J Respir Crit Care Med. 2020; 201:1380–88. https://doi.org/10.1164/rccm.202002-0445OC PMID:32275452

3. Komolafe O, Pereira SP, Davidson BR, Gurusamy KS. Serum c-reactive protein, procalcitonin, and lactate dehydrogenase for the diagnosis of pancreatic necrosis. Cochrane Database Syst Rev. 2017; 4:CD012645. https://doi.org/10.1002/14651858.CD012645 PMID:28431197

4. Van Wilpe S, Koornstra R, Den Brok M, De Groot JW, Blank C, De Vries J, Gerritsen W, Mehra N. Lactate dehydrogenase: a marker of diminished antitumor immunity. Oncoimmunology. 2020; 9:1731942. https://doi.org/10.1080/2162402X.2020.1731942 PMID:32158624

5. Pourfathi M, Cereda M, Chatterjee S, Xin Y, Kadlecek S, Duncan I, Hamedani H, Siddiqui S, Profka H, Ehrich J, Ruppert K, Rizi RR. Lung metabolism and inflammation during mechanical ventilation; an imaging approach. Sci Rep. 2018; 8:3525. https://doi.org/10.1038/s41598-018-21901-0 PMID:29476083

6. Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, Flemban H, Al-Nassir WN, Balkhy HH, Al-Hakeem RF, Makhdoom HQ, Zumla AI, Memish ZA. Epidemiological, demographic, and clinical characteristics of 47 cases of middle east respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. Lancet Infect Dis. 2013; 13:752–61. https://doi.org/10.1016/S1473-3099(13)70204-4 PMID:23891402

7. Mo P, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, Xiong Y, Cheng Z, Gao S, Liang K, Luo M, Chen T, Song S, et al. Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa270 PMID:32173725

8. Zhang R, Ouyang H, Fu L, Wang S, Han J, Huang K, Jia M, Song Q, Fu Z. CT features of SARS-CoV-2 pneumonia according to clinical presentation: a retrospective analysis of 120 consecutive patients from Wuhan city. Eur Radiol. 2020; 30:4417–26. https://doi.org/10.1007/s00330-020-06854-1 PMID:32279115

9. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

10. Li B, Yang J, Zhao F, Zhi L, Wang X, Liu L, Bi Z, Zhao Y. Prevalence and impact of cardiovascular metabolic diseases on COVID-19 in China. Clin Res Cardiol. 2020; 109:531–38. https://doi.org/10.1007/s00392-020-01626-9 PMID:32161990

11. Guo W, Li M, Dong Y, Zhou H, Zhang Z, Tian C, Qin R, Wang H, Shen Y, Du K, Zhao L, Fan H, Luo S, Hu D. Diabetes is a risk factor for the progression and prognosis of COVID-19. Diabetes Metab Res Rev. 2020. [Epub ahead of print]. https://doi.org/10.1002/dmrr.3319 PMID:32233013

12. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

13. Guan WJ, Liang WH, Zhao Y, Liang HR, Chen ZS, Li YM, Liu XQ, Chen RC, Tang CL, Wang T, Ou CQ, Li L, Chen PY, et al, and China Medical Treatment Expert Group for COVID-19. Comorbidity and its impact on 1590 patients with COVID-19 in China: a nationwide analysis. Eur Respir J. 2020; 55:2000547. https://doi.org/10.1183/13993003.00547-2020 PMID:32217650

14. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091. https://doi.org/10.1136/bmj.m1091 PMID:32217556

15. Liu Y, Yang Y, Zhang C, Huang F, Wang F, Yuan J, Wang Z, Li J, Li J, Feng C, Zhang Z, Wang L, Peng L, et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. Sci China Life Sci. 2020; 63:364–74. https://doi.org/10.1007/s11427-020-1643-8 PMID:32048163

16. Du RH, Liang LR, Yang CQ, Wang W, Cao TZ, Li M, Guo GY, Du J, Zheng CL, Zhu Q, Hu M, Li XY, Peng P, Shi HZ. Predictors of mortality for patients with COVID-19 pneumonia caused by SARS-CoV-2: a prospective cohort study. Eur Respir J. 2020; 55:2000524. https://doi.org/10.1183/13993003.00524-2020 PMID:32269088

17. Liu Y, Du X, Chen J, Jin Y, Peng L, Wang HH, Luo M, Chen L, Zhao Y. Neutrophil-to-lymphocyte ratio as an independent risk factor for mortality in hospitalized patients with COVID-19. J Infect. 2020; 81:e6–12. https://doi.org/10.1016/j.jinf.2020.04.002 PMID:32283162

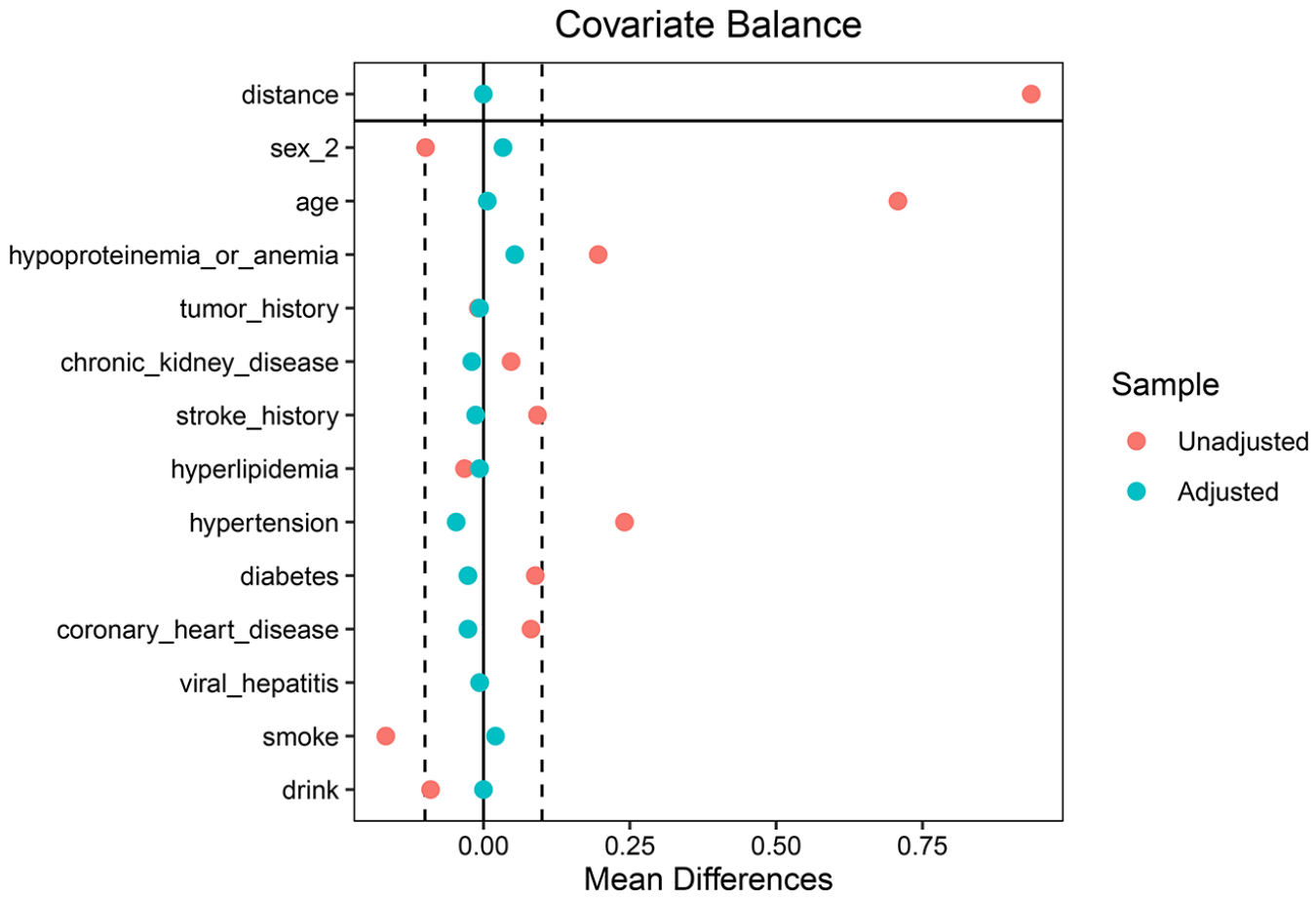
18. Duhamel A, Labreuche J, Gronnier C, Mariette C. Statistical tools for propensity score matching. Ann Surg. 2017; 265:E79–80. https://doi.org/10.1097/SLA.0000000000001312 PMID:28486297

19. Ding YQ, Bian XW. [Interpretation of pathological changes for "guidelines for the diagnosis and treatment of COVID-19 by the national health commission (trial version 7) "]. Zhonghua Bing Li Xue Za Zhi. 2020; 49:397–99. https://doi.org/10.3760/cma.j.cn112151-20200318-00221 PMID:32213267

20. Zhang Z. Propensity score method: a non-parametric technique to reduce model dependence. Ann Transl Med. 2017; 5:7. https://doi.org/10.21037/atm.2016.08.57 PMID:28164092

SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Mean differences in covariate balance before and after being adjusted.

Supplementary Tables

Supplementary Table 1. Laboratory indicators at admission between the nonsevere group and severe group.

	Nonsevere (n=128)	Severe (n=75)	P value*
WBC ($\times 10^9$/L)	5.61±2.16	7.17±3.99	0.002
Neutrophils ($\times 10^9$/L)	3.87±1.81	5.57±3.73	<0.001
Lymphocyte ($\times 10^9$/L)	1.23±0.67	1.01±0.45	0.014
NLR	3.93±3.17	7.2±6.41	<0.001
RBC ($\times 10^{12}$/L)	4.28±0.57	4.41±0.56	0.113
Platelet($\times 10^9$/L)	224.34±103.38	214.08±83.01	0.465
Albumin (g/L)	37.47±5.77	36.12±6.04	0.115
TBIL (μmol/L)	12.38±7.58	13.49±6.89	0.250
DBIL (μmol/L)	4.42±5.63	5.02±3.21	0.401
ALT (U/L)	35.49±32.48	35.61±29.96	0.980
AST (U/L)	33.54±22.04	37.60±22.39	0.209
Creatinine(μmol/L)	83.53±127.53	100.44±150.76	0.395
TG (mmol/L)	1.47±1.11	1.43±0.69	0.814
TC (mmol/L)	4.00±0.99	3.83±0.99	0.261
UA (μmol/L)	272.97±104.19	280.56±113.09	0.628
PCT (ng/mL)	0.20±0.70	0.32±0.90	0.296
CRP (mg/L)	31.84±49.83	75.52±73.09	<0.001
Fibrinogen (g/L)	3.99±1.45	4.65±1.36	0.002
D-dimer (μg/ml)	1.45±3.50	2.69±5.01	0.041
CK (U/L)	85.37±80.53	148.48±231.03	0.025
LDH (U/L)	215.23±97.36	349.28±177.60	<0.001

*Data were analyzed by Student's t-tests and Levene test was used to evaluate homogeneity of variance. Abbreviations: WBC, white blood cell; NLR, neutrophil-to-lymphocyte ratio; RBC, red blood cell; TBIL, total bilirubin; DBIL, direct bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; UA, uric acid; PCT, procalcitonin; CRP, c-reactive protein; CK, creatine kinase; LDH, lactic dehydrogenase.

Supplementary Table 2. Pearson correlation coefficient among levels of laboratory indicators.

	WBC	Neutrophils	Lymphocyte	CRP	fibrinogen	D-dimer	CK	LDH
WBC	1.00							
Neutrophils	0.96**	1.00						
Lymphocyte	0.22**	- 0.01	1.00					
CRP	0.31**	0.37**	- 0.37**	1.00				
Fibrinogen	0.13	0.21**	- 0.40**	0.54**	1.00			
D-dimer	0.17*	0.23**	- 0.20**	0.29**	0.04	1.00		
CK	0.02	0.00	- 0.08	0.20**	0.05	- 0.07	1.00	
LDH	0.34**	0.41**	- 0.36**	0.63**	0.34**	0.33**	0.40**	1

*There was a statistical difference at the level of P < 0.05. ** There was a statistical difference at the level of P < 0.01.
Abbreviations: WBC, white blood cell; CRP, C-reactive protein; CK, creatine kinase; LDH, lactic dehydrogenase.

Research Paper

Risk factors for severe cases of COVID-19: a retrospective cohort study

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ABSTRACT

Background: SARS-CoV-2 has raged around the world since March, 2020. We aim to describe the clinical characteristics and risk factors of severe patients with COVID-19 in Guangzhou.

Results: The severity and mortality of COVID-19 was 10.4% and 0.3% respectively. And each 1-year increase in age (OR, 1.057; 95% CI, 1.018-1.098; P=0.004), Wuhan exposure history greater than 2 weeks (OR, 2.765; 95% CI, 1.040-7.355; P=0.042), diarrhea (OR, 24.349; 95% CI, 3.580-165.609; P=0.001), chronic kidney disease (OR, 6.966; 95% CI, 1.310-37.058; P = 0.023), myoglobin higher than 106 μg/L (OR, 8.910; 95% CI, 1.225-64.816; P=0.031), white blood cell higher than 10×10^9/L (OR, 5.776; 95% CI, 1.052-31.722; P=0.044), and C-reactive protein higher than 10 mg/L (OR, 5.362; 95% CI, 1.631-17.626; P=0.006) were risk factors for severe cases.

Conclusion: Older age, Wuhan exposure history, diarrhea, chronic kidney disease, elevated myoglobin, elevated white blood cell and C-reactive protein were independent risk factors for severe patients with COVID-19 in Guangzhou.

Methods: We included 288 adult patients with COVID-19 and compared the data between severe and non-severe group. We used univariate and multivariate logistic regression methods to explore risk factors of severe cases.

INTRODUCTION

In December 2019, a large-scale infectious pneumonia of unknown origin broke out in Wuhan, China. Chinese scientists isolated a new coronavirus, SARS-CoV-2, causing the pneumonia on Jan 7, 2020 [1, 2]. And WHO named it Coronavirus Disease 2019 (COVID-19) in February 2020 [3]. Since March, justifying the previous data model [4], COVID-19 has raged across world. Up to Jun 3, 2020, there have been more than 6.4 million diagnosed cases in more than 200 countries, with a mortality rate of about 6% [5].

The clinical manifestations of COVID-19 range from mild to critical [6]. A lot of observational studies have described the clinical characteristics of patients with COVID-19 in Wuhan [7–10], but studies outside Wuhan have rarely been reported. Because of the virus variation, the clinical characteristics of the patients in Wuhan and outside Wuhan maybe different. In this study, we aimed to investigate patients with COVID-19 in Guangzhou to find their clinical characteristics and the risk factors for severe cases. Monitoring these factors can help clinicians identify severe patients early and take subsequent interventions to reduce their illness.

RESULTS

Baseline characteristics

Among the 288 patients, 30 cases were in severe group and only 1 case died by the end of the study. Thus, the severity and mortality were 10.4% and 0.3% respectively. The median age of all patients was 48.5 years (IQR 34.3-62), of which women accounted for 54.5% (Table 1). 134 (46.5%) patients had comorbidities, of which cardiovascular disease (CVD) (85, 29.5%) was the most common one, followed by hypertension (84, 29.2%), diabetes (24, 8.3%) (Table 1). 132 patients (45.8%) had a history of exposure to Wuhan 2 weeks before onset (Table 1). The most common symptoms on admission were fever (201, 69.8%) and cough (163, 56.6%), followed by sputum (58, 20.1%), fatigue (43, 14.9%), and myalgia (35, 12.2%) (Table 1).

Laboratory and radiological findings

216 (75%) patients had white blood cells (WBC) in normal range and lymphopenia occurred in 91 (31.6%) patients (Table 2). Compared with non-severe patients, severe patients had significantly reduced serum hemoglobin, platelet and myoglobin, as well as significantly increased WBC, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, creatine kinase, C-reactive protein (CRP), procalcitonin (PCT), brain natriuretic peptide (BNP), and troponin I (Table 2). 31 (10.8%) patients had unilateral pneumonia, and all of them were non-severe patients; 241 (83.7%) patients had bilateral pneumonia, of which 29 (96.7%) were severe patients (Table 2). Their chest CTs showed varying degrees of patchy ground-glass opacity, with lung lesion area of severe patients usually larger than that of non-severe patients (Figure 1).

Treatments and outcomes

244 (84.7%) patients received antibiotics, and 233 (80.9%) patients received antiviral drugs (oseltamivir / ribavirin; Table 3). There was a significant difference in the use of glucocorticoids and vasoactive drugs between non-severe and severe patients (Table 3). Five patients were treated with continuous renal replacement therapy (CRRT) and four patients were treated with extracorporeal membrane oxygenation (ECMO), and they were all severe patients (Table 3). 98.9% of the non-severe patients did not take oxygen or took normal-flux oxygen, while 43.3% of the severe patients took high-flux oxygen (Table 3). Eight patients were tracheal intubated and they were all severe patients (Table 3). Severe patients had a significant increase in use of non-invasive mechanical ventilation than non-severe patients (Table 3). Compared with non-severe patients, severe patients were more likely to be transferred to the intensive care unit (ICU), and suffer from ARDS, acute kidney injury and acute cardiac injury (Table 3).

Univariate and multivariate analysis of risk factors of severe cases

In univariate logistic regression analysis, we found that older patients with hypertension, chronic kidney disease (CKD), and a history of exposure in Wuhan were more likely to develop severe disease (Table 4). In addition, fever, shortness of breath, diarrhea, WBC, CRP, lymphocytes, COPD, CVD, hemoglobin, ALT, AST, myoglobin, creatinine, creatine kinase, PCT, BNP and TNI were also related with severe cases (Table 4).

The multivariable logistic regression model was constructed using all variables of significant statistical differences in univariate logistic regression analysis. We found that each 1-year increase in age (OR, 1.057; 95% CI, 1.018-1.098; P=0.004), Wuhan exposure history greater than 2 weeks (OR, 2.765; 95% CI, 1.040-7.355; P=0.042), CKD (OR, 6.966; 95% CI, 1.310-37.058; P = 0.023), diarrhea (OR, 24.349; 95% CI, 3.580-165.609; P=0.001), Myoglobin higher than 106 μg/L (OR, 8.910; 95% CI, 1.225-64.816; P=0.031), WBC higher than 10×10^9/L (OR, 5.776; 95% CI, 1.052-31.722; P=0.044), and CRP higher than 10 mg/L (OR, 5.362; 95% CI, 1.631-17.626; P=0.006) were independent risk factors for severe cases (Table 4).

DISCUSSION

Of the 288 patients in our database, only one case (0.3%) died, while the early mortality rate in Wuhan was as high as 28.3% [3]. And the severity of COVID-19 in Guangzhou is 10.4%, which was far less than that in early Wuhan of 31.7% [11].

It was interesting to note Guangzhou patients with Wuhan exposure history had a higher risk of becoming severe cases (Table 4). Earlier reports reported that some patients had SARS-CoV-2 gene fragments missing, suggesting that their virulence gradually weakened [12]. And an article reported that COVID-19 patients in Zhejiang Province had relatively mild symptoms compared with Wuhan [13]. Later, it was reported that SARS-CoV-2 has genomic diversity. It mutated through replication and may evolve under the pressure of immune surveillance in human body, with its virulence, infectivity and transmission being affected [14]. Therefore, the virulence of SARS-CoV-2 may increase or decrease during transmission, and certain populations in different regions may also have a screening effect on it, resulting in different disease

Table 1. Baseline characteristics of non-severe or severe patients of COVID-19 in Guangzhou.

Demographics and clinical characteristics	No. (%)			P value
	Total (288)	Non-severe (258)	Severe (30)	
Age, median (IQR), years	48.5 (34.3-62)	47 (33-61)	61.5(51-71.3)	<0.0001
Age groups (years):	..	..	..	<0.0001
≤30	44(15.3)	44(17.1)	0(0)	..
31-45	87(30.2)	83(32.2)	4(13.3)	..
46-65	116(40.3)	101(39.1)	15(50)	..
≥66	41(14.2)	30(11.6)	11(36.7)	..
Sex:	..	..	..	0.194
Male	131(45.5)	114(44.2)	17(56.7)	..
Female	157(54.5)	114(55.8)	13(43.3)	..
Comorbidity:	..	..	..	..
Hypertension	84(29.2)	69(26.7)	15(50)	0.008
SBP (mm Hg), median (IQR)	125(117-136)	125(117-136)	124.5(117-138.3)	0.186
DBP (mm Hg), median (IQR)	80(74-87)	80(75-87)	80.5(67.3-85)	0.028
MAP (mm Hg), median (IQR)	94.7(87.8-103)	94.7(88-103)	94.8(85.1-102.6)	0.415
Diabetes	24(8.3)	20(7.8)	4(13.3)	0.295
COPD	5(1.7)	3(1.2)	2(6.9)	0.025
CVD	85(29.5)	70(27.1)	15(50)	0.009
Carcinoma	6(2.1)	6(2.3)	0(0)	0.399
CKD	8(2.8)	4(1.6)	4(13.3)	<0.0001
CLD	10(3.5)	8(3.1)	2(6.7)	0.313
Exposure history in Wuhan >2 weeks:	..	..	..	0.016
Yes	132(45.8)	112(43.4)	20(66.7)	..
No	156(54.2)	146(56.6)	10(33.3)	..
Respiratory rate >24 breaths per min	19(6.6)	12(4.7)	7(23.3)	<0.0001
Oxygenation index, median (IQR)	98(97-98.8)	98(97-98.8)	98(97-99)	0.986
Fever (tempetature≥37·3°C)	201(69.8)	174(67.4)	27(90)	0.011
Cough	163(56.6)	142(55)	21(70)	0.118
Sputum	58(20.1)	54(20.9)	4(13.3)	0.326
Myalgia	35(12.2)	30(11.6)	5(16.7)	0.424
Fatigue	43(14.9)	37(14.3)	6(20)	0.410
Nausea or Anorexia	28(9.7)	22(8.5)	6(20)	0.045
Vomiting	6(2.1)	5(1.9)	1(3.3)	0.613
Diarrhea	11(3.8)	6(2.3)	5(16.7)	<0.0001
Headache	26(9)	22(8.5)	4(13.3)	0.385

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; MAP, Mean arterial pressure; COPD, Chronic obstructive pulmonary disease; CVD, Cardiovascular disease; CKD, Chronic kidney disease; CLD, Chronic liver disease.
***P* values** indicate differences between Severe and Non-severe patients. P<0.05 was considered statistically significant.

Table 2. Laboratory and radiological findings of non-severe or severe patients of COVID-19 in Guangzhou.

	Median (IQR)			P value	Normal range
	Total (288)	Non-severe (258)	Severe (30)		
Laboratory findings					
WBC (×10^9/L)	5.20(4.14-6.44)	5.14(4.10-6.38)	5.33(4.42-7.18)	0.934	4-10
WBC (×10^9/L) (No (%)):	..	..	..	<0.0001	..
<4	62(21.5)	57(22.1)	5(16.7)	..	..

4-10	216(75)	197(76.4)	19(63.3)	..	..
>10	10(3.5)	4(1.6)	6(20)	..	..
Lymphocyte count (×10^9/L)	1.42(1.04-1.96)	1.46(1.09-1.97)	1.03(0.84-1.38)	0.511	1.1-3.2
Lymphocyte count (×10^9/L) (No (%)):	..	..	..	..	..
<1.1	91(31.6)	73(28.3)	18(60)	<0.0001	
Hemoglobin (g/L)	135.5(123-147)	136(125-147.3)	123(114-143.3)	0.001	130-175
Platelet count (×10^9/L)	194.5(158-247)	199(160.1-249.3)	167(140.3-188.5)	0.043	125-350
D-dimer (mg/L)	1110(700-1700)	1090(680-1600)	1855(865-3442.5)	0.052	<1000
D-dimer (mg/L) (No (%)):	..	..	..	0.106	..
≤1000	125(43.9)	116(45.5)	9(30)	..	..
>1000	160(56.1)	139(54.5)	21(70)	..	..
ALT (U/L)	22.5(14.3-34.5)	22.1(14.2-33.9)	25(16.1-49.1)	0.912	9-50
ALT (U/L) (No (%)):	..	..	..	0.039	..
≤50	254(88.2)	231(89.5)	23(76.7)	..	..
>50	34(11.8)	27(10.5)	7(23.3)	..	..
AST(U/L)	18.4(14.9-25.6)	18.1(14.5-24.5)	21.9(16.8-41.1)	0.161	15-40
AST(U/L) (No (%)):	..	..	..	0.004	..
≤40	256(88.9)	234(90.7)	22(73.3)	..	..
>40	32(11.1)	24(9.3)	8(26.7)	..	..
Myoglobin (μg/L)	15(8.85-22.4)	14.4(8.6-21.2)	27.3(13.1-86.6)	0.212	17.4-105.7
Myoglobin (μg/L) (No (%)):	..	..	..	<0.0001	..
≤106	269(97.1)	247(99.2)	22(78.6)	..	..
>106	8(2.9)	2(0.8)	6(21.4)	..	..
Creatinine (μmol/L)	61.8(50.25-76.56)	62.0(50.4-76.4)	59.6(45.9-78.1)	0.428	54-106
Creatinine (μmol/L) (No (%)):	..	..	..	0.022	..
≤106	279(96.9)	252(97.7)	27(90)	..	..
>106	9(3.1)	6(2.3)	3(10)	..	..
Creatinine kinase (U/L)	52(36-80)	52(37-80)	44.5(27.5-128)	0.238	50-310
Creatinine kinase (U/L) (No (%)):	..	..	..	0.009	..
≤310	283(98.6)	255(99.2)	28(93.3)	..	..
>310	4(1.4)	2(0.8)	2(6.7)	..	..
CRP (mg/L)	9(8-22.72)	9(8-18.9)	24(11.7-51.2)	0.005	<10
CRP (mg/L) (No (%)):	..	..	..	<0.0001	..
≤10	175(60.8)	169(65.5)	6(20)	..	..
>10	113(39.2)	89(34.5)	24(80)	..	..
PCT (ng/mL)	0.13(0.04-32.6)	0.106(0.035-32.58)	0.2(0.09-51)	0.241	<0.05
PCT (ng/mL) (No (%)):	..	..	..	<0.0001	..
<0.05	99(35.5)	96(38.4)	3(10.3)	..	..
0.05-1.0	73(26.2)	56(22.4)	17(58.6)	..	..
1..0-10	6(2.2)	6(2.4)	0(0)	..	..
>10	101(36.2)	92(36.8)	9(31)	..	..
BNP (ng/L)	35(13-117.5)	18.5(9.75-40.25)	213(45-399)	0.014	<100
TNI (μg/L)	0.004(0.001-0.009)	0.003(0.001-0.007)	0.027(0.010-0.099)	0.033	<0.03
TNI (μg/L) (No (%)):	..	..	..	<0.0001	..
≤0.03	168(88.4)	159(92.4)	9(50)	..	..
>0.03	22(11.6)	13(7.6)	9(50)	..	..
Chest radiography findings					
Unilateral pneumonia	31(10.8)	31(12.1)	0(0)	0.044	..
Bilateral pneumonia	241(83.7)	212(82.2)	29(96.7)	0.042	..

WBC, White blood cell; ALT, Alanine transaminase; AST, Aspartate aminotransferase; CRP, C-reactive protein; PCT, Procalcitonin; BNP, Brain natriuretic peptide; TNI, Troponin I.

***P* values** indicate differences between Severe and Non-severe patients. P<0.05 was considered statistically significant.

degrees and influencing factors of COVID-19 in different regions.

According to previous reports, older age was an important independent predictor of SARS and MERS mortality [15, 16]. Previous studies have confirmed increased severity and mortality of COVID-19 in old patients [3, 7, 17]. A recent study comparing the clinical characteristics and results of COVID-19 patients of different ages showed that the symptoms of elderly patients were more atypical, with more comorbidities, secondary infection, organ injuries, immunodeficiency and a higher risk of critical illness [18]. Many comorbidities in the elderly such as hypertension, diabetes and CKD were treated with ACE inhibitors and angiotensin II receptor blockers, which would upregulate the ACE2 receptor, thereby increasing the risk of SARS-CoV-2 infection and the risk of disease [19]. In our study cohort, age was also one of the risk factors for severe patients (Table 4). Therefore, it's very important for old patients to have early diagnosis and treat systemic comorbidities carefully.

SARS-CoV-2 was reported to be detected in stool samples from patients [20], and a study of a family cluster have reported two COVID-19 patients who had only diarrhea symptom [9]. Besides diarrhea, some patients also had other gastrointestinal symptoms such as vomiting and abdominal pain [21]. Our analysis showed that diarrhea was a risk factor for severe cases (Table 4), which suggested that beside of damaging the respiratory system, the virus may also have a certain function on the digestive system. This finding may be related to the expression of SARS-CoV-2 receptor ACE2 in both the epithelial cells of lungs and digestive tract [21, 22]. Given the small number of diarrhea cases

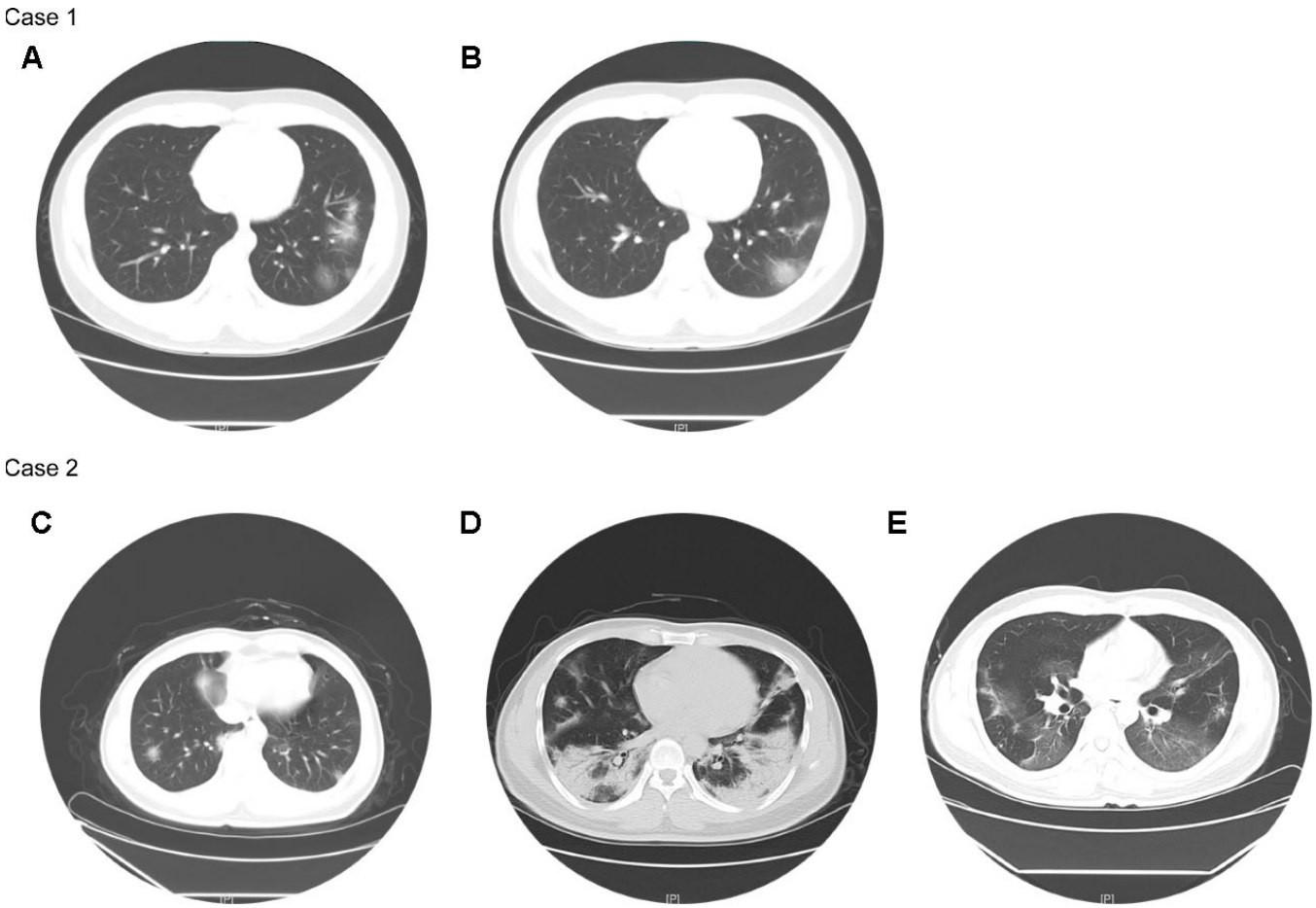

Figure 1. Chest CTs of two representative cases. Case 1 (non-severe): Chest CT on Feb 24 (**A**) showed multiple patchy ground-glass opacity in both lungs, with unclear borders and uneven density. Chest CT on Feb 28 (**B**) showed better status, and some lesions were slightly absorbed than before. Case 2 (severe): Chest CT on Jan 29 (**C**) showed the texture of both lungs was slightly increased, and both lungs were scattered in patchy shadows, whose edges were blurred. Chest CT on Feb 11 (**D**) showed the scope of the bilateral lung lesions was enlarged, the density was increased, and the local consolidation and bronchial signs were seen. Chest CT on Mar 4 (**E**) showed improved status, and both lung lesions were significantly less than before.

Table 3. Treatments and outcomes of non-severe or severe patients of COVID-19 in Guangzhou.

	No. (%)			P value
	Total (288)	Non-severe (258)	Severe (30)	
Treatments				
Antiviral	233(80.9)	204(79.1)	29(96.7)	0.020
Antibiotics	244(84.7)	214(82.9)	30(100)	0.014
Vasoactive drugs	5(1.7)	1(0.4)	4(13.3)	<0.0001
Glucocorticoid	21(7.3)	12(4.7)	9(30)	<0.0001
CRRT	5(1.7)	0(0)	5(16.7)	<0.0001
ECMO	4(1.4)	0(0)	4(13.3)	<0.0001
Oxygen uptake:	..	..	..	..
None	88(30.6)	84(32.6)	4(13.3)	0.030
Normal-flux	184(63.9)	171(66.3)	13(43.3)	0.013
High-flux	16(5.6)	3(1.2)	13(43.3)	<0.0001
Tracheal intubation	8(2.8)	0(0)	8(26.7)	<0.0001
Tracheotomy	0(0)	0(0)	0(0)	..
Non-invasive mechanical ventilation	32(11.1)	13(5)	19(63.3)	<0.0001
Outcomes				
ICU Admission	27(9.4)	12(4.7)	15(50)	<0.0001
ARDS	3(1)	0(0)	3(10)	<0.0001
Acute kidney injury	5(1.7)	0(0)	5(16.7)	<0.0001
Acute cardiac injury	22(11.6)	13(7.6)	9(50)	<0.0001

CRRT, continuous renal-replacement therapy; ECMO, Extracorporeal membrane oxygenation; ICU, intensive care unit; ARDS, Acute respiratory distress syndrome.

***P* values** indicate differences between Severe and Non-severe patients. P<0.05 was considered statistically significant.

Table 4. Univariate and multivariate analysis of risk factors of severe cases in Guangzhou.

	Univariable OR (95% CI)	P value	Multivariable OR (95%) CI)	P value
Demographics and clinical characteristics				
Age, years	1.063(1.033-1.095)	<0.0001	1.057 (1.018-1.098)	0.004
Female sex (vs male)	0.605(0.282-1.298)	0.197	..	..
Comorbidity present (vs not present)				
Hypertension	2.739(1.272-5.898)	0.010	..	..
COPD	6.296(1.007-39.354)	0.049	..	..
CVD	2.686(1.248-5.780)	0.012	0.986(0.052-18.588)	0.992
CKD	9.769(2.307-41.376)	0.002	6.966(1.310-37.058)	0.023
Respiratory rate >24 breaths per min	6.239(2.238-17.397)	<0.0001	..	..
Exposure history in Wuhan >2 weeks	2.607(1.174-5.791)	0.019	2.765(1.040-7.355)	0.042
Fever (tempetature≥37·3°C)	4.345(1.282-14.730)	0.018	..	..
Nausea or Anorexia	2.682(0.991-7.258)	0.052	..	..
Diarrhea	8.400(2.392-29.494)	0.001	24.349(3.580-165.609)	0.001
Laboratory and radiography findings				
White blood cell count (10^9/L) (No (%)):	..	..	..	..
≤4	0.910(0.325-2.543)	0.857	0.968(0.289-3.245)	0.958
4-10	1(ref)	..	..	..
≥10	15.553(4.032-59.988)	<0.0001	5.776(1.052-31.722)	0.044
Lymphocyte count (×10^9/L)	..	..	..	..
<1.1	0.263(0.121-0.573)	0.001	0.697(0.246-1.975)	0.497

Hemoglobin (g/L)	0.966(0.946-0.986)	0.001	..	..
Platelet count ($\times 10^9$/L)	0.993(0.987-1.000)	0.042	..	..
D-dimer (mg/L)	..	..	..	..
≤1000	1(ref)	..	..	..
>1000	1.947(0.859-4.416)	0.111	..	..
ALT (U/L)	..	..	..	..
≤50	1(ref)	..	..	..
>50	2.604(1.022-6.634)	0.045	..	..
AST(U/L)	..	..	..	..
≤40	1(ref)	..	..	..
>40	3.545(1.425-8.823)	0.007	..	..
Myoglobin (μg/L)	..	..	..	..
≤106	1(ref)	..	..	..
>106	33.682(6.413-176.905)	<0.0001	8.910(1.225-64.816)	0.031
Creatinine (μmol/L)	..	..	..	..
≤106	1(ref)	..	..	..
>106	4.667(1.104-19.728)	0.036	..	..
Creatinine kinase (U/L)	..	..	..	..
≤310	1(ref)	..	..	..
>310	9.107(1.234-67.188)	0.030	..	..
CRP (mg/L)	..	..	..	..
≤10	1(ref)	..	..	..
>10	7.596(2.995-19.264)	<0.0001	5.362(1.631-17.626)	0.006
PCT (ng/mL)	..	..	..	..
≤0.05	1(ref)	..	..	..
>0.05	5.333(1.572-18.098)	0.007	..	..
BNP (ng/L)	1.022(1.005-1.040)	0.014	..	..
TNI (μg/L)	..	..	..	..
≤0.03	1(ref)	..	..	..
>0.03	12.231(4.14-36.131)	<0.0001	..	..
Bilateral pneumonia	6.292(0.836-47.378)	0.074	..	..

OR=odds ratio.

***P* values** indicate differences between Severe and Non-severe patients. P<0.05 was considered statistically significant.

(11, 3.8%) (Table 1), SARS-CoV-2-induced digestive system damage may also be related to other physical factors of these patients, which deserves further study.

Previous studies have reported that COVID-19 non-survivors had more neutrophil counts than survivors, which may be related to cytokine storms caused by virus invasion [7, 11]. Our analysis found that elevated WBC and CRP were risk factors for severe cases (Table 4). Like neutrophils, WBC and CRP are also indicators of inflammatory status in the body. When they elevated, there may be a cytokine storm caused by virus invasion in the body, which may cause severe inflammation in lungs and other organs, and aggravate the disease. Therefore, paying close attention to changes in WBC, CRP and making timely correction can effectively reduce the number of severe cases and deaths.

In our study, myoglobin, creatine kinase, BNP, and TNI were increased in severe patients compared to non-severe patients (Table 2), and myoglobin was a risk factor for severe patients, which indicated that COVID-19 may be related to acute cardiac injury. ACE2 is also expressed in heart [23], and SARS-CoV has been shown in animal models to directly mediate myocardial inflammation and damage by down-regulating myocardial ACE2 and lead to poor cardiac prognosis [24]. A meta-analysis involving 4189 patients showed that more severe COVID-19 was associated with increased troponin, creatine kinase, myoglobin, and NT-proBNP [25]. Myoglobin was also included in the COVID-19 severity score table as one of the biomarkers [26]. The severity of COVID-19 may be related to acute cardiac injury, which prompts us to effectively monitor heart condition to prevent

COVID-19 patients from myocarditis and avoid poor cardiac prognosis.

Many studies have reported that comorbidities were major risk factors for increasing COVID mortality and poor prognosis [7, 8], and CKD was one of them. Due to older age, previous comorbidities, impaired immune system, and regular visits to crowded outpatient dialysis centers, CKD patients have increase susceptibility to SARS-COV-2 [27]. On one hand, the above factors have greatly reduced the ability of CKD patients to overcome the virus and may lead to severe disease or even death. On the other hand, SARS-COV-2 can directly damage kidney by combine with ACE2 [28], and cause kidney inflammation and acute kidney injury [13, 29], which was consistent with the increase creatinine level of severe patients in our study. AKI could further aggravate CKD as well as worsening the patients' whole conditions, leading patients to develop severe illness.

The study has several limitations. First, the sample size of our study cohort was relatively small including only 288 patients from a single center. Due to the exploratory nature of the study, which was not driven by formal hypotheses, we did not estimate the sample size, but included as many cases as possible. Second, this study lacked laboratory data such as serum cytokines and chemokines, so that we cannot evaluate the inflammation levels and cytokine storms of these patients. Third, this was a retrospective study. The data in this study was only a preliminary assessment of clinical characteristics and risk factors of COVID-19 severe patients. Further researches are still needed.

In conclusion, our research showed that the severity and mortality of COVID-19 in Guangzhou were much lower than those in early Wuhan. The risk factors for severe cases of COVID-19 in Guangzhou included older age, Wuhan exposure history greater than 2 weeks, diarrhea, elevated Myoglobin, elevated WBC and CRP, and CKD. Investigating and monitoring these factors can help clinicians identify patients with poor prognosis at an early stage, and take proactive interventions to benefit patients and reduce severity and mortality. It also provided significant experience and reference for countries around the world to fight against COVID-19.

MATERIALS AND METHODS

Study design and participants

This single-center, retrospective cohort study was conducted at Guangzhou Eighth People's Hospital (Guangzhou, China), which was the designated hospital to treat patients with COVID-19 in Guangzhou. From Jan 15, 2020 to Mar 10, 2020, we recruited 288 adult patients with COVID-19 (the total number was 292, including 4 underage patients).

This study was approved by the Ethics Committee of Guangzhou Eighth People's Hospital, and informed consent was obtained from all patients enrolled.

Definitions

According to the Chinese diagnosis and treatment guideline for COVID-19 (trial version 7.0) [6], 288 patients were divided into non-severe group (258 cases), including light and general patients, and severe group (30 cases), including severe and critical patients. A case was defined as severe if it met any of the following: (1) shortness of breath, respiratory rate ≥ 30 times / minute; (2) blood oxygen saturation ≤ 93% at rest; (3) oxygenation index (PaO2 / FiO2) ≤ 300 mmHg; (4) pulmonary infiltrates > 50% of the lung lesions within 24-48 hours; (5) respiratory failure, requiring mechanical ventilation; (6) shock; (7) combine with multiple organ dysfunction, needing ICU monitoring treatment. Acute Respiratory Distress Syndrome (ARDS) was defined according to WHO's guidance for COVID-19 [30]. Acute renal injury (ARI) was determined from serum creatinine [31]. Acute cardiac injury (ACI) was determined based on the serum concentration of troponin I (TNI) [11]. The reference ranges of all laboratory inspection indicators were measured in the laboratory of Guangzhou Eighth People's Hospital.

Data collection

This study reviewed the clinical electronic medical records, nursing records, laboratory tests and radiological findings of 288 adult patients with COVID-19, who were confirmed by nucleic acid testing. And we extracted epidemiology, demographics, clinical manifestations, laboratory data, chest radiography findings, treatment and outcome data for statistical analysis and research.

Statistical analysis

The purpose of this study was to analyze the risk factors of severe patients by comparing severe group and non-severe group in terms of their clinical data. Therefore, no formal assumptions were used to facilitate the calculation of the sample size, and we included the largest number of patients who met the inclusion criteria.

We represented continuous variables as median and interquartile range (IQR), and categorical variables as

frequency (N) and percentage (%). We assessed differences between severe group and non-severe group using two-sample *t* test or the Mann-Whitney U test depending on parametric or nonparametric data for continuous variables, and χ 2 test or Fisher's exact test for categorical variables. Univariate and multivariate logistic regression models were used to explore the risk factors for severe cases.

A P value of less than 0.05 was considered statistically significant. All data were statistically analyzed using SPSS software (version 25).

AUTHOR CONTRIBUTIONS

Dr. Feng He, Dr. Qingqing Luo and Dr. Ming Lei contributed equally, they all developed conceptualization and wrote the manuscript under the supervision of Dr. Na Yu, Dr. Liuping Yang and Dr. Jie Cao. Lixin Fan, Xinning Shao, Guanglie Huang, Jun Zeng, Ziwen Zhao, Shuguang Qin, Zhi Yang has participated substantially in the conceptualization and design of this work as well as the writing of the manuscript. All authors have reviewed the final version of the manuscript and have approved it for publication.

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CONFLICTS OF INTEREST

We declare no conflicts of interest.

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Editorial note

&This corresponding author has a verified history of publications using the personal email addresses for correspondence.

REFERENCES

1. Phelan AL, Katz R, Gostin LO. The novel coronavirus originating in Wuhan, China: challenges for global health governance. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.1097 PMID:31999307

2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

3. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

4. Wu JT, Leung K, Leung GM. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. Lancet. 2020; 395:689–97. https://doi.org/10.1016/S0140-6736(20)30260-9 PMID:32014114

5. COVID-19 cases worldwide. June 3, 2020. https://www.worldometers.info/coronavirus/

6. National Health Commission of the People's Republic of China. Chinese diagnosis and treatment guideline for COVID-19 (trial version 7.0). March 4, 2020. http://www.nhc.gov.cn/xcs/zhengcwj/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml

7. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

8. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

9. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, Xing F, Liu J, Yip CC, Poon RW, Tsoi HW, Lo SK, Chan KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. Lancet. 2020; 395:514–23.

https://doi.org/10.1016/S0140-6736(20)30154-9 PMID:31986261

10. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

11. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

12. Su YCF, Anderson DE, Young BE, Linster M, Zhu F, Jayakumar J, Zhuang Y, Kalimuddin S, Low JGH, Tan CW, Chia WN, Mak TM, Octavia S, et al. Discovery and Genomic Characterization of a 382-Nucleotide Deletion in ORF7b and ORF8 during the Early Evolution of SARS-CoV-2. mBio. 2020; 11:e01610–20. https://doi.org/10.1128/mBio.01610-20 PMID:32694143

13. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, Li SB, Wang HY, Zhang S, Gao HN, Sheng JF, Cai HL, Qiu YQ, Li LJ. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-cov-2) outside of Wuhan, China: retrospective case series. BMJ. 2020; 368:m606. https://doi.org/10.1136/bmj.m606 PMID:32075786

14. Shen Z, Xiao Y, Kang L, Ma W, Shi L, Zhang L, Zhou Z, Yang J, Zhong J, Yang D, Guo L, Zhang G, Li H, et al. Genomic diversity of SARS-CoV-2 in coronavirus disease 2019 patients. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa203 PMID:32129843

15. Hong KH, Choi JP, Hong SH, Lee J, Kwon JS, Kim SM, Park SY, Rhee JY, Kim BN, Choi HJ, Shin EC, Pai H, Park SH, Kim SH. Predictors of mortality in middle east respiratory syndrome (MERS). Thorax. 2018; 73:286–89. https://doi.org/10.1136/thoraxjnl-2016-209313 PMID:28724637

16. Choi KW, Chau TN, Tsang O, Tso E, Chiu MC, Tong WL, Lee PO, Ng TK, Ng WF, Lee KC, Lam W, Yu WC, Lai JY, Lai ST, and Princess Margaret Hospital SARS Study Group. Outcomes and prognostic factors in 267 patients with severe acute respiratory syndrome in hong kong. Ann Intern Med. 2003; 139:715–23. https://doi.org/10.7326/0003-4819-139-9-200311040-00005 PMID:14597455

17. Li P, Chen L, Liu Z, Pan J, Zhou D, Wang H, Gong H, Fu Z, Song Q, Min Q, Ruan S, Xu T, Cheng F, Li X. Clinical features and short-term outcomes of elderly patients with COVID-19. Int J Infect Dis. 2020; 97:245–50. https://doi.org/10.1016/j.ijid.2020.05.107 PMID:32492533

18. Zhao M, Wang M, Zhang J, Gu J, Zhang P, Xu Y, Ye J, Wang Z, Ye D, Pan W, Shen B, He H, Liu M, et al. Comparison of clinical characteristics and outcomes of patients with coronavirus disease 2019 at different ages. Aging (Albany NY). 2020; 12:10070–86. https://doi.org/10.18632/aging.103298 PMID:32499448

19. Shahid Z, Kalayanamitra R, McClafferty B, Kepko D, Ramgobin D, Patel R, Aggarwal CS, Vunnam R, Sahu N, Bhatt D, Jones K, Golamari R, Jain R. COVID-19 and older adults: what we know. J Am Geriatr Soc. 2020; 68:926–29. https://doi.org/10.1111/jgs.16472 PMID:32255507

20. Zhang H, Kang Z, Gong H, Xu D, Wang J, Li Z, Cui X, Xiao J, Meng T, Zhou W, Liu J, Xu H. The digestive system is a potential route of 2019-nCov infection: a bioinformatics analysis based on single-cell transcriptomes. bioRxiv. https://doi.org/10.1101/2020.01.30.927806

21. Wong SH, Lui RN, Sung JJ. Covid-19 and the digestive system. J Gastroenterol Hepatol. 2020; 35:744–48. https://doi.org/10.1111/jgh.15047 PMID:32215956

22. Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, Geng Q, Auerbach A, Li F. Structural basis of receptor recognition by SARS-CoV-2. Nature. 2020; 581:221–24. https://doi.org/10.1038/s41586-020-2179-y PMID:32225175

23. Zou X, Chen K, Zou J, Han P, Hao J, Han Z. Single-cell RNA-seq data analysis on the receptor ACE2 expression reveals the potential risk of different human organs vulnerable to 2019-nCoV infection. Front Med. 2020; 14:185–92. https://doi.org/10.1007/s11684-020-0754-0 PMID:32170560

24. Oudit GY, Kassiri Z, Jiang C, Liu PP, Poutanen SM, Penninger JM, Butany J. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. Eur J Clin Invest. 2009; 39:618–25. https://doi.org/10.1111/j.1365-2362.2009.02153.x PMID:19453650

25. Li JW, Han TW, Woodward M, Anderson CS, Zhou H, Chen YD, Neal B. The impact of 2019 novel coronavirus on heart injury: a systematic review and meta-analysis. Prog Cardiovasc Dis. 2020; S0033-0620:30080–83. https://doi.org/10.1016/j.pcad.2020.04.008 PMID:32305557

26. McRae MP, Simmons GW, Christodoulides NJ, Lu Z, Kang SK, Fenyo D, Alcorn T, Dapkins IP, Sharif I, Vurmaz D, Modak SS, Srinivasan K, Warhadpande S, et al. Clinical decision support tool and rapid point-of-care platform for determining disease severity in patients with COVID-19. Lab Chip. 2020; 20:2075–85. https://doi.org/10.1039/d0lc00373e PMID:32490853

27. Jung HY, Lim JH, Kang SH, Kim SG, Lee YH, Lee J, Chang HH, Kim SW, Choi JY, Cho JH, Kim CD, Kim YL, Park SH. Outcomes of COVID-19 among patients on in-center hemodialysis: an experience from the epicenter in South Korea. J Clin Med. 2020; 9:E1688. https://doi.org/10.3390/jcm9061688 PMID:32498262

28. Zhang F, Liang Y. Potential risk of the kidney vulnerable to novel coronavirus 2019 infection. Am J Physiol Renal Physiol. 2020; 318:F1136–37. https://doi.org/10.1152/ajprenal.00085.2020 PMID:32223555

29. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G. China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020; 382:1708–1720. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

30. WHO Clinical management of severe acute respiratory infection when novel coronavirus (nCoV) infection is suspected. Jan 11, 2020. https://www.who.int/publications-detail/clinical-management-of-severe-acute-respiratory-infection-when-novel-coronavirus-(ncov)-infection-is-suspected

31. Khwaja A. KDIGO clinical practice guidelines for acute kidney injury. Nephron Clin Pract. 2012; 120:c179–84. https://doi.org/10.1159/000339789 PMID:22890468

Research Paper

The effect of emergency surgery on acute abdomen patients with COVID-19 pneumonia: a retrospective observational study

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ABSTRACT

During the COVID-19 outbreak, some patients with COVID-19 pneumonia also suffered from acute abdomen requiring surgical treatment; however, there is no consensus for the treatment of such patients. In this study, we retrospectively reviewed 34 patients with acute abdomen who underwent emergency surgery during the COVID-19 outbreak. Among the 34 patients with acute abdomen, a total of six cases were found with COVID-19 pneumonia (clinical classification for COVID-19 pneumonia: all were the common type). On the premise of similar demographics between both groups, patients with COVID-19 pneumonia had worse indicators of liver and coagulation function. Compared with acute abdomen patients without COVID-19, patients with COVID-19 pneumonia had a longer hospital stay, but there were no significant differences in postsurgical complications (P = 0.58) or clinical outcomes (P = 0.56). In addition, an obvious resolution of lung inflammation after surgery was observed in five COVID-19 patients (83.3%). No new COVID-19 cases occurred during the patients' hospital stays. Therefore, for the common type of COVID-19 pneumonia, emergency surgery could not only improve the outcomes of COVID-19 pneumonia patients with acute abdomen, but also benefit the resolution of pulmonary inflammation.

INTRODUCTION

In the last two decades there have been two large-scale pandemics caused by coronaviruses, severe acute respiratory syndrome (SARS) [1] and Middle East respiratory syndrome (MERS) [2]. At the end of 2019, another novel coronavirus, designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan and subsequently spread rapidly throughout the world [3, 4]. Due to accumulating evidence of continuous person-to-person transmission and a general susceptibility of humans to the virus [5–7], the World Health Organization (WHO) declared coronavirus disease 2019 (COVID-19) a public health emergency of international concern on January 30, 2020. As of May 16, 2020, COVID-19 caused 309,713 deaths among over 4.5 million patients across more than 200 countries, with a case-fatality rate of 6.8%. Although SARS-CoV-2 was found to predominantly infect the

lower airways and cause life-threatening pneumonia [8, 9], evidence has revealed that the digestive system might be another potential viral target [7, 10, 11].

Acute abdomen is defined as acute onset of abdominal pain which requires accurate diagnosis and treatment within a particular time limit to prevent mortality and morbidity [12]. During COVID-19 outbreaks, some patients with COVID-19 pneumonia also suffered from acute abdomen requiring immediate interventions [13]. However, previous studies have demonstrated that preoperative pneumonia is a significant risk factor for poor postsurgical outcomes [14, 15]. In addition, surgical treatment might increase medical staff exposure to SARS-CoV-2 [16, 17] and trigger excessive inflammation in the patient, resulting in worsening of COVID-19 pneumonia [18]. Therefore, an investigation of the impact of emergency surgery on patients with both acute abdomen and COVID-19 pneumonia is urgently needed.

RESULTS

Clinical characteristics of patients

Among the 34 patients with acute abdomen who underwent emergency surgery, six patients had COVID-19, and the remaining 28 patients did not. The baseline characteristics of all patients are summarized in Table 1. No new infections were found in medical staff or patients throughout the hospitalization period.

Of the 28 patients who did not have COVID-19 pneumonia (9 female and 19 male; mean age 55 years (range 17–87)), 12 (43%) patients were diagnosed with acute appendicitis, 10 (36%) with gastrointestinal perforation, 5 (18%) with intestinal obstruction and 1 (4%) with bladder rupture. The typical abdominal CT appearance is shown in Figure 1. Comorbidities were found in 17 (61%) patients and included diabetes mellitus in 7 (25%), coronary heart disease in 7 (25%), hypertension in 6 (21.4%), chronic obstructive pulmonary disease in 2 (7.1%), chronic renal failure in 1 (3.6%), chronic liver failure in 1 (3.6%), acute myeloid leukemia in 1 (3.6%), and rheumatoid arthritis in 1 (3.6%). Five (17.9%) patients were reported to have postoperative complications: one had intra-abdominal infection, one had a wound infection, and three had multiple organ dysfunction syndrome (MODS). All three patients with postoperative MODS had preoperative comorbidities (case 1: coronary heart disease and chronic renal failure; case 2: chronic liver failure; case 3: hypertension and coronary heart disease). In total, 25 (89.3%) patients were cured, and the remaining 3 patients died due to severe septic shock and MODS.

The detailed clinical characteristics of the six patients with both acute abdomen and COVID-19 pneumonia are shown in Table 2. Age of the six patients (4 women and 2 men) ranged from 66 to 78 years. Three patients were diagnosed with intestinal obstruction, two with acute appendicitis, and one with gangrenous cholecystitis. The clinical classification of COVID-19 pneumonia in all patients was the common type. Three patients had hypertension, and one had coronary heart disease. The most common clinical manifestations were abdominal pain and fever. Two (33.3%) patients tested positive for SARS-CoV-2 by RT-PCR, and one patient tested positive for IgM-IgG antibodies; however, typical CT imaging manifestations of COVID-19 pneumonia were found in all six patients. Postoperative complications occurred in two patients: one had aspiration pneumonia and the other had MODS. All patients received antiviral therapy (ribavirin, 500 mg each time, twice times a day, 5-7 days; arbidol, 200 mg each time, three times a day, 5-7 days; Interferon α-2b, 5.0×10^5 IU, nebulized inhalation, twice times a day) and antibacterial therapy, and four patients received immunoglobulins (human immunoglobulin, 10g/d). Two patients with postoperative complications received mechanical ventilation and systematic corticosteroid treatment (methylprednisolone, 1–2 mg/kg.d, 3–5 days). In total, five patients were cured, and one patient died of postoperative MODS.

Emergency surgery could not only improve the outcomes of acute abdomen patients with COVID-19 pneumonia, but also benefit the resolution of pulmonary inflammation

The baseline characteristics in patients with and without COVID-19 pneumonia are shown in Table 1. Differences in demographics, including age ($P = 0.12$), sex ($P = 0.17$), diagnosis ($P = 0.06$) and comorbidities ($P = 0.67$), between both groups were not significant. However, patients with COVID-19 pneumonia had higher ALT (70.7 ± 108.3 U/L vs. 18.7 ± 7 U/L, $P = 0.012$), AST (72.7 ± 93.7 U/L vs. 20.6 ± 13.7 U/L, $P = 0.006$), APTT (50.7 ± 10 s vs. 36.1 ± 3.6 s, $P < 0.001$), and PT (16.9 ± 4.5 s vs. 14.1 ± 1.2 s, $P = 0.006$), and lower albumin (30 ± 10.8 g/L vs. 41.6 ± 6.5 g/L, $P = 0.012$) and hemoglobin (107.2 ± 26.8 g/L vs. 143.9 ± 17.4 g/L, $P < 0.001$) than patients who did not have COVID-19 pneumonia. In addition, although there were no significant differences, patients with COVID-19 pneumonia had lower infection-related biomarkers, including WBC ($(10.4 \pm 6.5)\times10^9$/L vs. $(11.8 \pm 3.8)\times10^9$/L, $P = 0.49$), lymphocyte ($(0.7 \pm 0.3)\times10^9$/L vs. $(1.1 \pm 0.7)\times10^9$/L, $P = 0.26$), neutrophil ($(8.9 \pm 5.9)\times10^9$/L vs. $(10.1 \pm 3.5)\times10^9$/L, $P = 0.51$), CRP (82.6 ± 72.9 mg/L vs.

Table 1. The baseline characteristics of all patients with acute abdomen.

Characteristics	Patients with acute abdomen		P-value
	With COVID-19 (n = 6)	Without COVID-19 (n = 28)	
Age (years)	70 ± 4.2	55 ± 22	0.120
Gender			0.170
Female	4 (67%)	9 (32%)	
Male	2 (33%)	19 (68%)	
Diagnosis			0.060
Acute appendicitis	2 (33%)	12 (43%)	
Gastrointestinal perforation	0 (0%)	10 (36%)	
Intestinal obstruction	3 (50%)	5 (18%)	
Gangrenous cholecystitis	1 (17%)	0 (0%)	
Bladder rupture	0 (0%)	1 (4%)	
Comorbidities			0.670
No	3 (50%)	11 (39%)	
Yes	3 (50%)	17 (61%)	
Laboratory findings			
WBC ($\times 10^9$/L)	10.4 ± 6.5	11.8 ± 3.8	0.490
Neutrophil($\times 10^9$/L)	8.9 ± 5.9	10.1 ± 3.5	0.510
Lymphocyte ($\times 10^9$/L)	0.7 ± 0.3	1.1 ± 0.7	0.260
HGB (g/L)	107.2 ± 26.8	143.9 ± 17.4	<0.001
CRP (mg/L)	82.6 ± 72.9	139.2 ± 67.1	0.074
PCT (μg/L)	3.4 ± 5.3	8.8 ± 8.7	0.160
Albumin (g/L)	30 ± 10.8	41.6 ± 6.5	0.001
ALT (U/L)	70.7 ± 108.3	18.7 ± 7	0.012
AST (U/L)	72.7 ± 93.7	20.6 ± 13.7	0.006
D-Dimer (mg/L)	2.6 ± 3.3	1.4 ± 1.2	0.140
APTT (s)	50.7 ± 10	36.1 ± 3.6	<0.001
PT (s)	16.9 ± 4.5	14.1 ± 1.2	0.006

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; APTT, activated partial thromboplastin time; PT, prothrombin time; HGB, hemoglobin; WBC, white blood cell; CRP, C-reaction protein; PCT, procalcitonin.

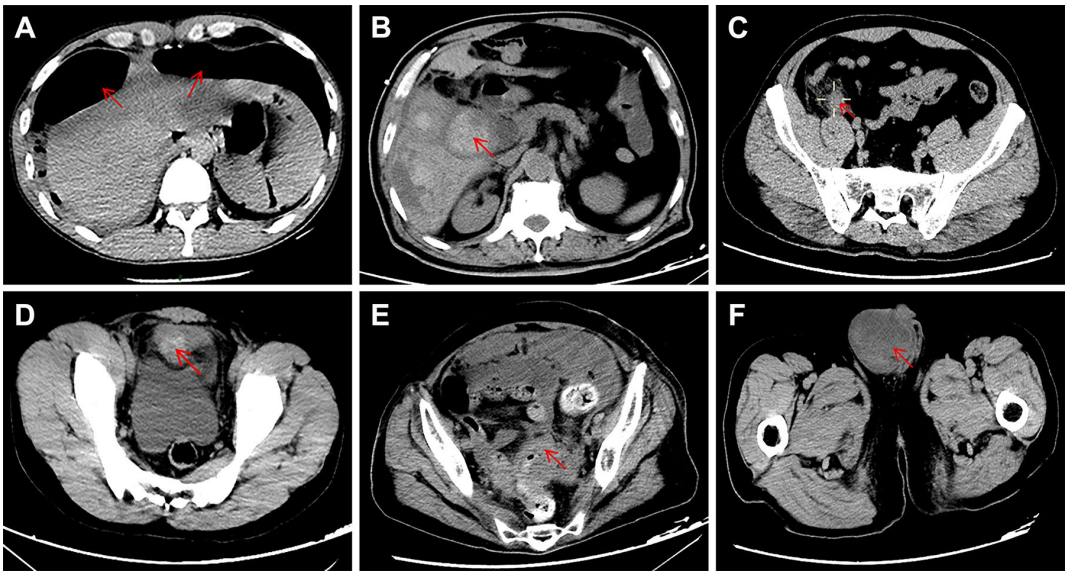

Figure 1. Typical appearance of abdominal CT showing the causes of acute abdomen in the present study. (**A**) duodenal perforation accompanied by free intraperitoneal gas; (**B**) gangrenous cholecystitis; (**C**) acute appendicitis; (**D**) bladder rupture; (**E**) intestinal obstruction caused by carcinomas in the rectosigmoid junction; (**F**) intestinal obstruction caused by inguinal incarcerated hernia.

Table 2. The clinical characteristics of the six patients with both acute abdomen and COVID-19 pneumonia.

Characteristics	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age, years	69	78	68	68	66	69
Gender	Female	Male	Female	Male	Female	Female
Evidence of COVID-19						
RT-PCR	Negative	Positive	Negative	Negative	Negative	Positive
IgM-IgG antibodies	NA	NA	NA	NA	Negative	Positive
Typical CT manifestation	Unilateral	Bilateral	Bilateral	Unilateral	Bilateral	Bilateral
Diagnosis	Intestinal volvulus Pneumonia (mild)	Gangrenous cholecystitis Pneumonia (mild)	Acute appendicitis Pneumonia (mild)	Malignant intestinal obstruction Pneumonia (mild)	Acute appendicitis Pneumonia (mild)	Malignant intestinal obstruction Pneumonia (mild)
Symptoms and signs						
Fever	No	Yes	Yes	Yes	Yes	No
Cough	Yes	No	No	Yes	No	No
Expectoration	No	Yes	No	Yes	No	Yes
Abdominal pain	Yes	Yes	Yes	Yes	Yes	Yes
Diarrhea	No	No	No	No	Yes	No
Nausea and vomiting	Yes	Yes	No	Yes	No	Yes
Comorbidities	No	Hypertension	No	Hypertension, CHD	Hypertension	No
Postoperative complications	No	MODS	No	Aspiration pneumonia	No	No
Treatment						
Mechanical ventilation	No	Yes	No	Yes	No	No
Antibiotics	Yes	Yes	Yes	Yes	Yes	Yes
Antivirals	Yes	Yes	Yes	Yes	Yes	Yes
Immune globulins	Yes	Yes	No	Yes	Yes	No
Hormones	No	Yes	No	Yes	No	No
Clinical outcome	Discharged	Death	Discharged	Discharged	Discharged	Discharged

Abbreviations: NA, not available; CHD, coronary heart disease; MODS, multiple organ dysfunction syndrome.

139.2 ± 67.1 mg/L, P = 0.074) and PCT (3.4 ± 5.3 μg/L vs. 8.8 ± 8.7 μg/L, P = 0.16), than patients who did not have COVID-19 pneumonia.

The comparative data of postsurgical outcomes between the two groups are shown in Figure 2. Compared with patients who did not have COVID-19 pneumonia, patients with COVID-19 pneumonia had a longer hospital stay (19.3 ± 10 days vs. 10.4 ± 6.6 days, P = 0.009), but no significant differences in postsurgical complications (P = 0.58) and clinical outcomes (P = 0.56) were found between groups. Furthermore, the majority of worsening preoperative laboratory indicators, including ALT (P = 0.43), AST (P = 0.93), APTT (P = 0.1), PT (P = 0.14), albumin (P = 0.44) and hemoglobin (P = 0.06), had improved by the third postoperative day. As outlined in Figure 3, when compared with preoperative indicators, postoperative infection-related biomarkers also decreased, including WBCs ((10.4 ± 6.5)×10^9/L vs. (5.4 ± 3.2)×10^9/L, P = 0.19), neutrophils ((8.9 ± 5.9)×10^9/L vs. (3.9 ± 3.4)×10^9/L, P = 0.16), CRP (82.6 ± 72.9 mg/L vs. 56.1 ± 49.8 mg/L, P = 0.55) and PCT (3.4 ± 5.3 μg/L vs. 0.3 ± 0.2 μg/L, P = 0.29).

To remove the potential impact of age on the above results, we further compared the pre- and postsurgical differences between patients with COVID-19 and those without COVID-19 pneumonia (between 60 and 80 years old). After age-matching between both groups, the majority of preoperative and postoperative results were consistent with the previous results. As

shown in Table 3, patients with COVID-19 pneumonia still had poor preoperative liver and coagulation function. However, the bulk of abnormal preoperative laboratory findings were significantly and rapidly corrected after surgical treatment (Figure 4). In addition, an obvious resolution of lung inflammation was observed after surgery in five patients (83.3%) (Figure 5). These results indicated that COVID-19 pneumonia is associated with poor liver function and coagulation function in acute abdomen patients with COVID-19 pneumonia. Nevertheless, emergency surgery could not only improve the outcomes of

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Characteristics	Patients with acute abdomen		P value
	With COVID-19 (n = 6)	Without COVID-19 (n = 28)	
Length of hospitalization (days)	19.3 ± 10	10.4 ± 6.6	0.009
Postoperative complications			0.580
No	4 (67%)	23 (82%)	
Yes	2 (33%)	5 (18%)	
Clinical outcome			0.560
Discharged	5 (83%)	25 (89%)	
Death	1 (17%)	3 (11%)	

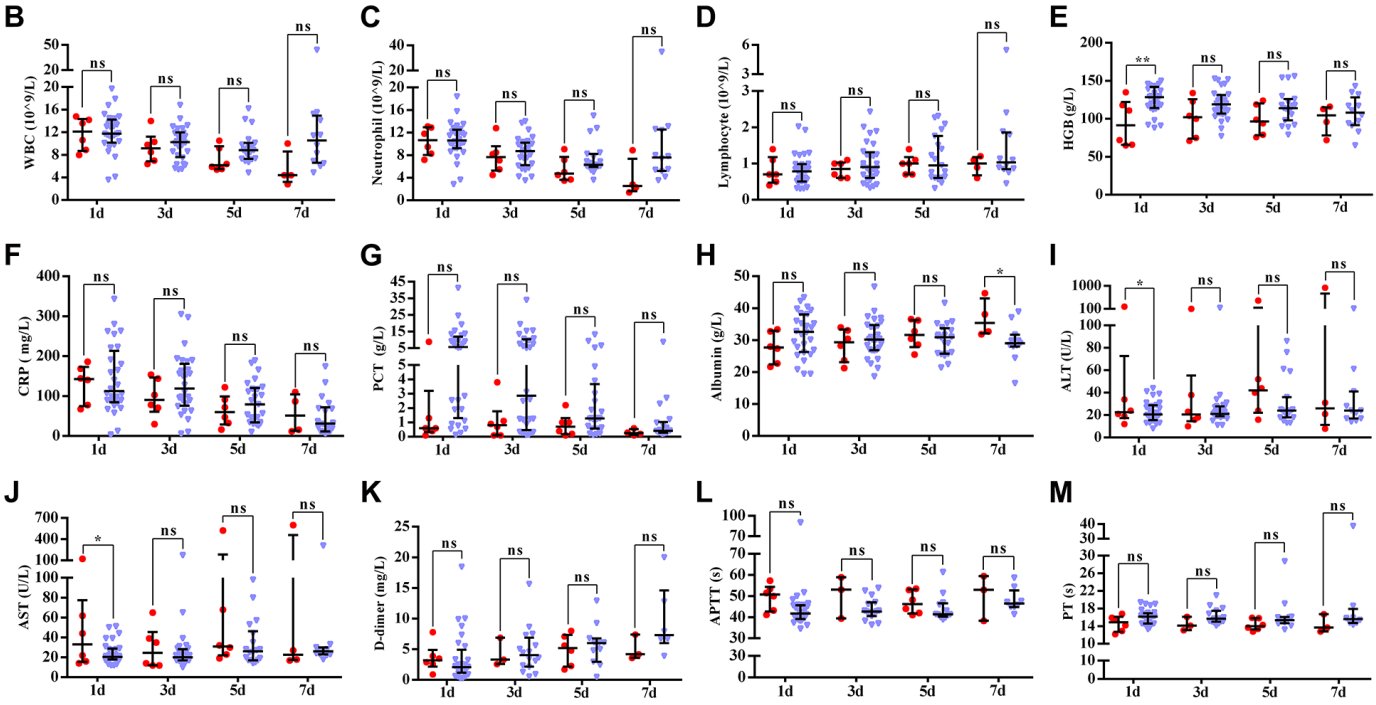

Figure 2. Postoperative outcomes of all patients with acute abdomen. Data are presented as numbers and percentages for categorical variables, and continuous data are expressed as the mean ± standard deviation (SD). *P< 0.05, **P< 0.01, ***P <0.001, based on Student's t-test. (**A**) the difference between both groups in clinical outcomes; (**B**–**M**) shows the differences between patients with and without COVID-19 pneumonia in postoperative laboratory findings, including (**B**) WBCs (white blood cells); (**C**) neutrophils; (**D**) lymphocytes; (**E**) HGB (hemoglobin); (**F**) CRP (C-reactive protein); (**G**) PCT (procalcitonin); (**H**) Albumin; (**I**) ALT (alanine aminotransferase); (**J**) AST (aspartate aminotransferase); (**K**) D-dimer; (**L**) APTT (activated partial thromboplastin time); (**M**) PT (prothrombin time). Red and blue marks represent patients with and without COVID-19 pneumonia, respectively.

COVID-19 pneumonia patients with acute abdomen, but also benefit the resolution of pulmonary inflammation.

DISCUSSION

Accurate diagnosis and appropriate intervention within a particular time limit is crucial to prevent deterioration and mortality in patients with acute abdomen [12]. Although previous studies revealed that preoperative pneumonia is significantly associated with worse postoperative outcomes [14, 15], there is still no direct evidence suggesting that surgical treatment leads to adverse effects in acute abdomen patients with COVID-19 pneumonia. Using the data from 34 patients with acute abdomen who underwent emergency surgery at our institute, the results of our study show that COVID-19 pneumonia is associated with poor liver function and coagulation function in acute abdomen patients with COVID-19 pneumonia. However, emergency surgery could not only improve the outcomes of COVID-19 pneumonia patients with acute abdomen, but also benefit the resolution of pulmonary inflammation.

COVID-19 might complicate the perioperative course of acute abdomen [13, 19]. The bulk of evidence revealed that SARS-CoV-2 RNA was identified in stool specimens [7, 20] and that the viral receptor angiotensin-converting enzyme 2 (ACE2) was highly expressed in gastrointestinal epithelial cells [21, 22], this evidence supported the conclusion that the digestive system is a potential target of SARS-CoV-2. In addition, infection-related biomarkers (including peripheral blood lymphocytes and WBCs) tend to decrease in patients with COVID-19 pneumonia [3, 4], while these indicators frequently increase in patients who only have acute abdomen. Blanco-Colino et al. also reported a case of suspected acute abdomen as an extrapulmonary manifestation of COVID-19 [19]. All of these results demonstrated that COVID-19 likely interferes with the accurate diagnosis and clinical assessment of acute abdomen.

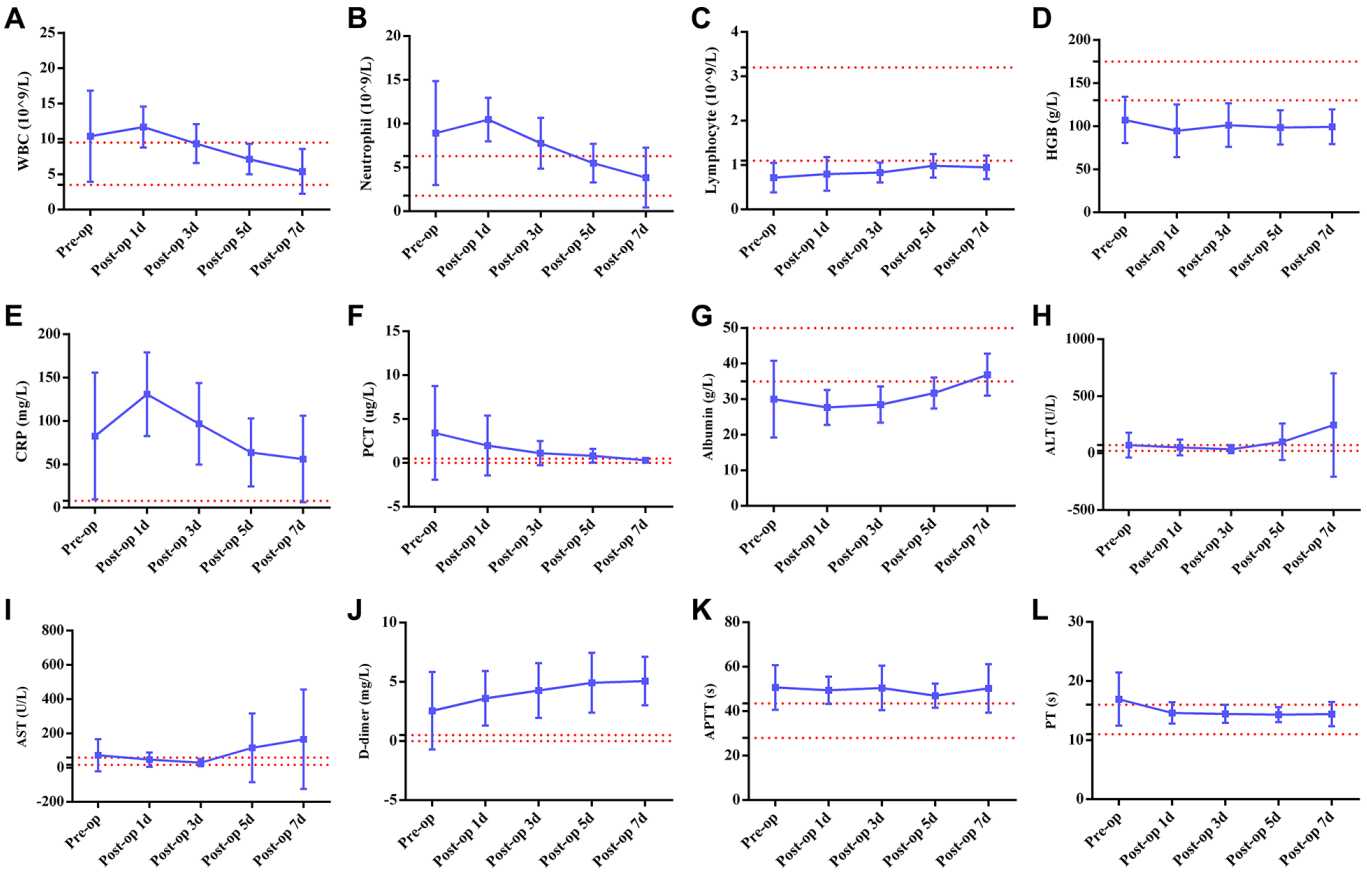

Figure 3. Line graphs illustrating detailed changes in laboratory findings in six patients with both acute abdomen and COVID-19 pneumonia. The red line represents the normal range of laboratory findings. (**A**) WBCs (white blood cells); (**B**) neutrophils; (**C**) lymphocytes; (**D**) HGB (hemoglobin); (**E**) CRP (C-reactive protein); (**F**) PCT (procalcitonin); (**G**) Albumin; (**H**) ALT (alanine aminotransferase); (**I**) AST (aspartate aminotransferase); (**J**) D-dimer; (**K**) APTT (activated partial thromboplastin time); (**L**) PT (prothrombin time).

Table 3. The preoperative differences between patients with COVID-19 pneumonia and those without COVID-19 pneumonia after age-matching.

Characteristics	Patients with acute abdomen		P-value
	With COVID-19 (n = 6)	Without COVID-19 (n = 12)	
Age (years)	70 ± 4.2	71.2 ± 5.9	0.590
Gender			0.620
Female	4 (67%)	5 (42%)	
Male	2 (33%)	7 (58%)	
Diagnosis			0.110
Acute appendicitis	2 (33%)	5 (42%)	
Gastrointestinal perforation	0 (0%)	5 (42%)	
Intestinal obstruction	3 (50%)	2 (17%)	
Gangrenous cholecystitis	1 (17%)	0 (0%)	
Comorbidities			0.340
No	3 (50%)	3 (25%)	
Yes	3 (50%)	9 (75%)	
Laboratory findings			
WBC ($\times 10^9$/L)	10.4 ± 6.5	9.6 ± 2.6	0.730
Neutrophil($\times 10^9$/L)	8.9 ± 5.9	8.3 ± 2.4	0.750
Lymphocyte ($\times 10^9$/L)	0.7 ± 0.3	1.0 ± 0.7	0.300
HGB (g/L)	107.2 ± 26.8	143.6 ± 13.2	0.001
CRP (mg/L)	82.6 ± 72.9	148.9 ± 79.7	0.110
PCT (μg/L)	3.4 ± 5.3	11.9 ± 11.5	0.110
Albumin (g/L)	30 ± 10.8	38.3 ± 5.2	0.040
ALT (U/L)	70.7 ± 108.3	15.3 ± 4.8	0.086
AST (U/L)	72.7 ± 93.7	15.8 ± 6.3	0.046
D-Dimer (mg/L)	2.6 ± 3.3	1.4 ± 1.1	0.290
APTT (s)	50.7 ± 10	37.2 ± 4.4	<0.001
PT (s)	16.9 ± 4.5	14.1 ± 0.8	0.042

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; APTT, activated partial thromboplastin time; PT, prothrombin time; HGB, hemoglobin; WBC, white blood cell; CRP, C-reaction protein; PCT, procalcitonin.

To better carry out emergency surgery during the outbreak, our hospital has developed a detailed management strategy for acute abdomen patients. For patients with stable vital signs and local involvement (such as acute appendicitis alone, acute cholecystitis alone, and incomplete ileus) not requiring emergency surgery, conservative treatment in the outpatient department can be considered. If conservative treatment fails, emergency surgery should be performed immediately. The goal of emergency surgery is to remove the patient's lesions rapidly and effectively while minimizing the operation time and limiting the medical staff's exposure.

The indications for emergency surgery should be strictly managed during the COVID-19 outbreak. The possible reasons for opposing surgical interventions for acute abdomen accompanied with COVID-19 pneumonia are as follows: 1) Surgical interventions on patients with COVID-19 may lead to contamination of the operating room and surgical equipment and risk transmission of the infection to healthcare providers and other patients in the hospital [17, 23]; 2) surgical treatment may trigger oxidative stress [24] and immunosuppression [25], which might hinder the clearance of SARS-CoV-2 and accelerate the progression of COVID-19 pneumonia. However, the scientific foundation of this theory is very weak. Jamali et al. reported that preoperative pneumonia only moderately increased the risk of mortality (OR= 1.2) in patients undergoing emergency surgery [14]. Moreover, an improvement of acute abdomen and pneumonia after surgery was observed in our study. A possible explanation for such results is that surgical

treatment alleviated excessive inflammation and persistent immunosuppression caused by acute abdomen, which in turn contributed to clearance of the virus and resolution of lung inflammation. In addition, medical staff could effectively prevent SARS-CoV-2 infection through adherence to strict infection prevention and control protocols [16, 26]. No new infections were found in medical staff or patients throughout the hospitalization of patients with or without COVID-19 pneumonia in our study.

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Characteristics	Patients with acute abdomen		P value
	With COVID-19 (n = 6)	Without COVID-19 (n = 12)	
Length of hospitalization (days)	19.3 ± 10	13.3 ± 5.2	0.032
Postoperative complications			0.792
No	4 (67%)	9 (75%)	
Yes	2 (33%)	3 (25%)	
Clinical outcome			1.000
Discharged	5 (83%)	10 (83%)	
Death	1 (17%)	2 (17%)	

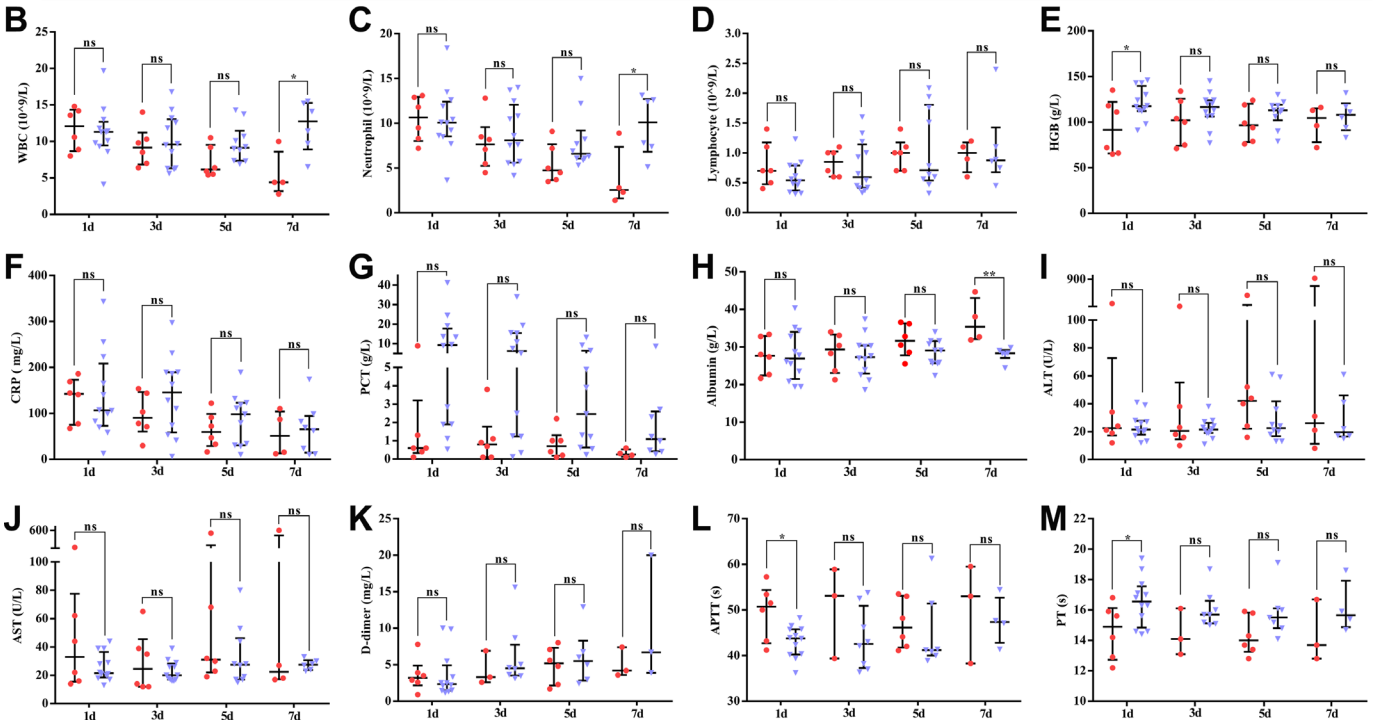

Figure 4. The difference between patients with COVID-19 pneumonia and those without COVID-19 pneumonia (aged between 60 and 80) in postoperative outcomes. Data are presented as numbers and percentages for categorical variables, and continuous data are expressed as the mean ± standard deviation (SD). *P< 0.05, **P< 0.01, ***P <0.001, based on Student's t-test. (**A**) The difference between both groups in clinical outcomes; (**B**–**M**) shows the differences in postoperative laboratory findings between patients with and without COVID-19 pneumonia, including (**B**) WBCs (white blood cells); (**C**) neutrophils; (**D**) lymphocytes; (**E**) HGB (hemoglobin); (**F**) CRP (C-reactive protein); (**G**) PCT (procalcitonin); (**H**) Albumin; (**I**) ALT (alanine aminotransferase); (**J**) AST (aspartate aminotransferase); (**K**) D-dimer; (**L**) APTT (activated partial thromboplastin time); (**M**) PT (prothrombin time). Red and blue marks represent patients with and without COVID-19 pneumonia, respectively.

Current clinical observations have found that most COVID-19 patients have fever and acute abdomen patients often have fever. In our study, 5 patients (17.9%) presented with fever before emergency surgery. Some postoperative patients may present with fever, which may result from postoperative traumatic stress or residual abdominal infection. This makes it extremely difficult to identify the cause of fever and to identify COVID-19 in a timely manner. Elderly patients, especially those with pulmonary infections, are more susceptible to COVID-19 during the postoperative hospitalization period. Therefore, we monitored the patient's body temperature closely, and routine blood parameters, including PCT and CRP, were regularly retested. If necessary, a chest CT scan was performed again to monitor COVID-19 pneumonia progression. To ensure therapeutic efficacy, we streamlined treatments to reduce doctor-patient contact and avoid cross-infection.

This study had certain limitations that should be discussed. First, due to the lack of definite practical guidance for patients with both acute abdomen and COVID-19 pneumonia, the indication and timing of the surgical treatment was decided empirically instead of being based on evidence. Second, this was a small-sample nonrandomized retrospective study without strict inclusion and exclusion criteria, and as such, there were potential biases that could affect the comparison analysis. Third, the availability of clinical care and the diversity of COVID-19 management may limit the applicability of our results. However, to our knowledge, the results of our study provide the first evidence that emergency surgery could not only improve the outcomes of acute abdomen patients with COVID-19 pneumonia, but also benefit the resolution of pulmonary inflammation. These results hopefully lead to a consensus on the treatment and management of acute abdomen patients with or without COVID-19 during the COVID-19 outbreak.

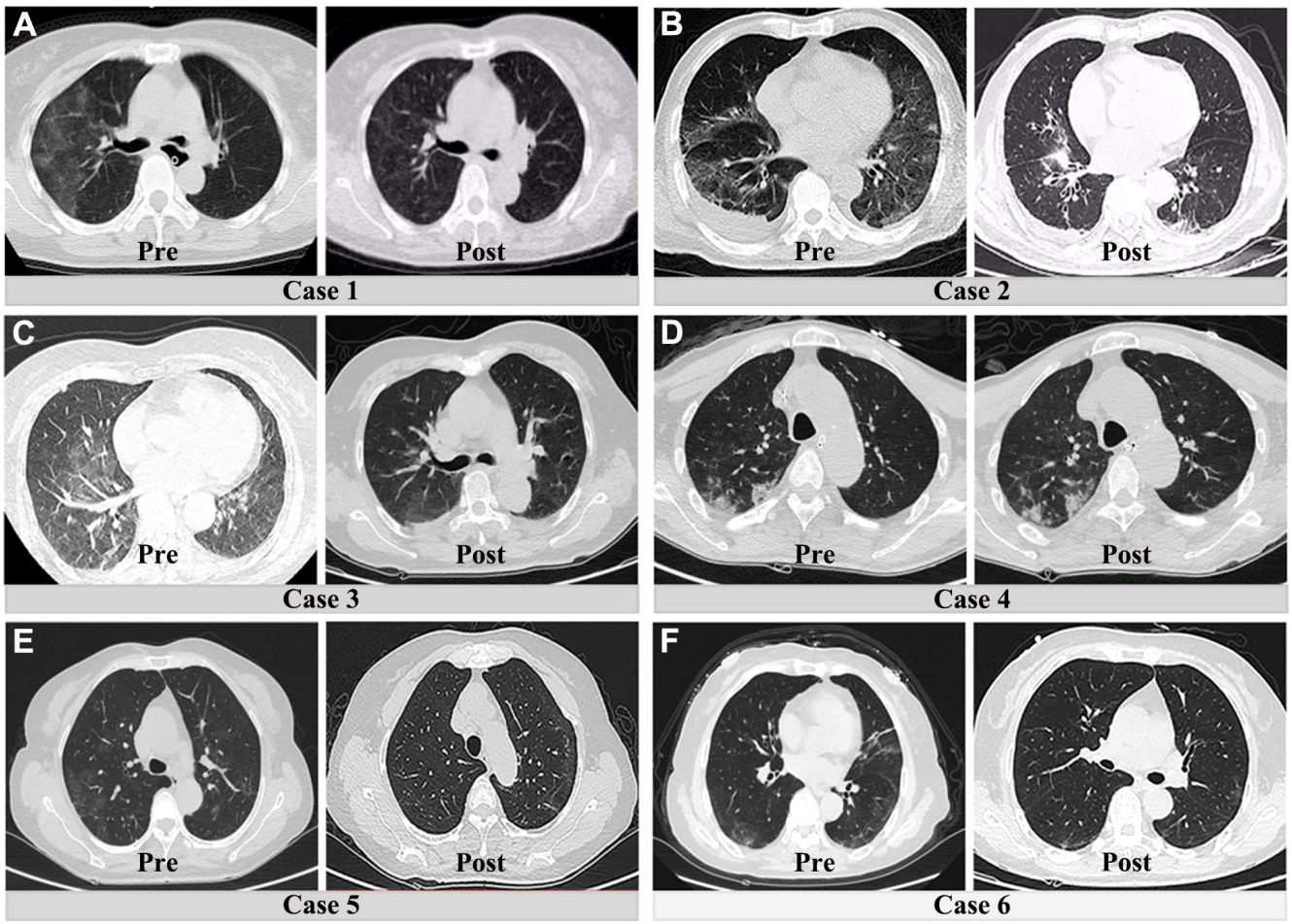

Figure 5. Preoperative and postoperative CT lung manifestations in six patients with both acute abdomen and COVID-19 pneumonia. (**A**–**C**) and (**E**, **F**) show the obvious resolution of pulmonary inflammation. The fourth patient had no significant change of pulmonary inflammation after surgical treatment (**D**).

MATERIALS AND METHODS

Study design and patient cohort

We retrospectively reviewed 34 patients with acute abdomen who underwent emergency surgery from February 2, 2020, to March 18, 2020, at the Union Hospital affiliated with Tongji Medical College, Huazhong University of Science and Technology. This study protocol was approved by the ethics committee of our college. All patients signed an informed consent document indicating their understanding of the procedure and its potential complications as well as their approval to participate in the research study. A flow diagram of the emergency surgery protocol for patients with acute abdomen during COVID-19 outbreaks is presented in Figure 6.

Preoperative work-up

After a detailed history and a complete physical examination, all patients with acute abdomen underwent routine laboratory testing (such as complete blood counts, serum biochemistry and tumor-marker screening) and imaging examination (such as chest X-ray, abdominal ultrasound, contrast-enhanced computed tomography (CT)). Prior to admission, all patients also completed a detailed risk assessment for COVID-19, including typical clinical manifestation and contact history with suspicious or confirmed COVID-19 patients within 14 days. CT lung imaging, quantitative reverse transcription-polymerase chain reaction (RT-PCR) and IgM-IgG antibodies against SARS-CoV-2 were also required for all patients to screen for potential infections. All suspected COVID-19 cases were treated as positive until confirmed. If emergency surgery was required, patients with positive indicators for infection must be taken directly to a designated COVID operation room through a predefined path. Due to the possible false negatives of test kits, all surgical procedures were carried out using a high level of protection, including masks, eye protection, gloves, caps and protective clothing, for the entire duration of the procedure.

Postoperative work-up

All patients who were not excluded from possibly having COVID-19 were transferred to the isolation ward and transitional ward after surgery according to the status of the preoperative screening results. Medical staff were required to adhere to strict prevention and infection control protocols in addition to routine universal precautions, and all patients were advised to wear a mask throughout hospitalization. Patients in the transitional ward underwent another round of RT-PCR for SARS-CoV-2. If the screening yielded negative results, the patient was transferred and treated in a single room of the general ward for three to five days prior to transfer to a shared room. If patients presented with pyrexia of unknown origin, typical respiratory symptoms or CT imaging manifestations indicating viral pneumonia, they were transferred to the

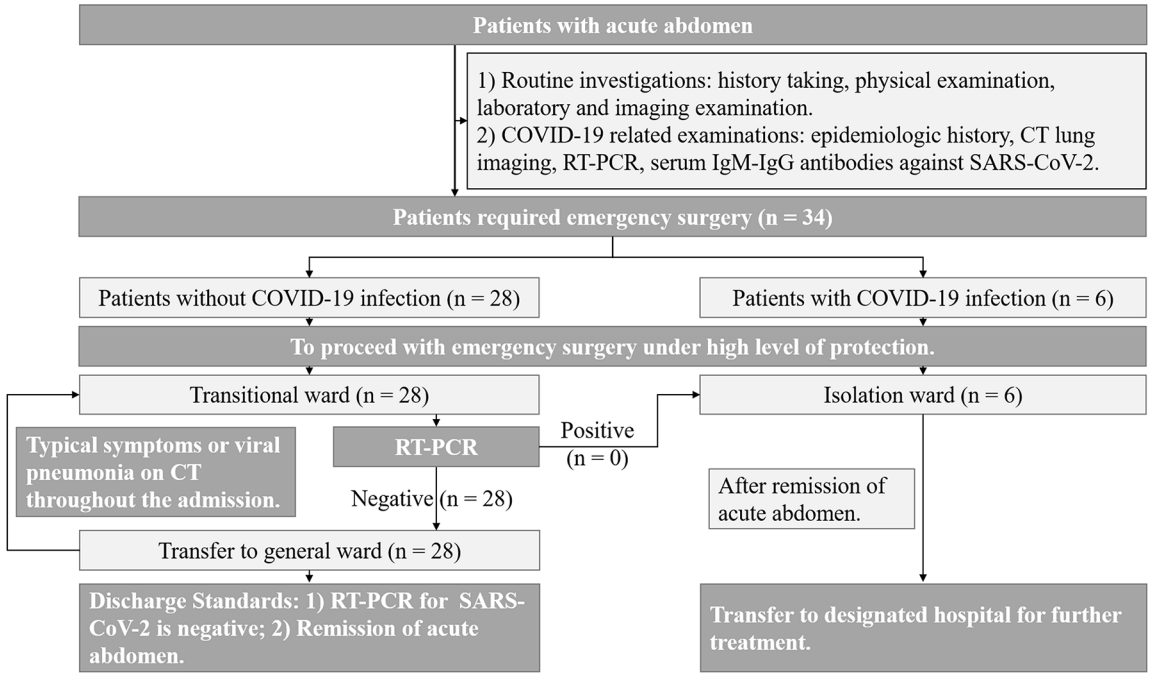

Figure 6. Flow diagram for performing emergency surgery for acute abdomen patients during COVID-19 outbreak.

transitional ward to retest for SARS-CoV-2 infection by RT-PCR. If the screening yielded positive results, patients were transferred to the isolation ward for further treatment. After the remission of acute abdomen, patients in the isolation ward were advised to transfer to designated hospitals for the treatment of COVID-19.

Data collection

Primary data, including clinical characteristics, the laboratory and imaging examination results, evidence of COVID-19, treatments, and clinical outcomes, were identified from medical reports. Blood samples from all participants were obtained through peripheral venipuncture. The following thresholds were considered the normal range of indicators: creatinine, 58-110 μmol/L; total bilirubin, 3-22 μmol/L; albumin, 35-50 g/L; alanine aminotransferase (ALT), 21-72 U/L; aspartate aminotransferase (AST), 17-59 U/L; hemoglobin (HGB), 130-175 g/L; white blood cell (WBC) count, $(3.5\text{-}9.5)\times10^9$/L; neutrophil count, $(1.8\text{-}6.3)\times10^9$/L; lymphocyte count, $(1.1\text{-}3.2)\times10^9$/L; C-reaction protein (CRP), < 8 mg/L; procalcitonin (PCT), < 0.5 μg/L; D-dimer, < 0.5 mg/L; activated partial thromboplastin time (APTT), 28-43.5 s; and prothrombin time (PT), 11-16 s. We adopted the classification system of the New Coronavirus Pneumonia Prevention and Control Program (7th edition). According to this system, COVID-19 pneumonia cases were divided into four groups: mild, moderate, severe and critically ill. The discharge requirements for patients who only had acute abdomen include 1) remission of acute abdomen and 2) negative SARS-CoV-2 results by RT-PCR. However, for acute abdomen patients with COVID-19 pneumonia, obvious resolution of pulmonary inflammation and negative results by RT-PCR for two consecutive evaluation times were necessary for discharge.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA), and diagrams of curves were drawn using Prism (version 6.0; GraphPad). Data are presented as numbers and percentages for categorical variables, and continuous data are expressed as the mean ± standard deviation (SD). Differences were considered significant at *p<0.05, **p <0.01 and ***p <0.001. ns: no significance.

AUTHOR CONTRIBUTIONS

All authors conceived the study and study design, iteratively drafted the article, and approved the final article. ZG and CJH obtained institutional review board approval. CYF, ZH, HP, HCJ, CD, XP, CQY and CP coordinated the data collection. ZN, WL, and CYF coordinated discussions with statisticians and performed the analyses. ZG and CJH take responsibility for the paper as a whole.

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CONFLICTS OF INTEREST

The authors have declared that no conflicts of interest exist.

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REFERENCES

1. Zhong NS, Zheng BJ, Li YM, Poon LL, Xie ZH, Chan KH, Li PH, Tan SY, Chang Q, Xie JP, Liu XQ, Xu J, Li DX, et al. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. Lancet. 2003; 362:1353–58. https://doi.org/10.1016/s0140-6736(03)14630-2 PMID:14585636

2. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012; 367:1814–20. https://doi.org/10.1056/NEJMoa1211721 PMID:23075143

3. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

4. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

5. Chan JF, Yuan S, Kok KH, To KK, Chu H, Yang J, Xing F, Liu J, Yip CC, Poon RW, Tsoi HW, Lo SK, Chan KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person

transmission: a study of a family cluster. Lancet. 2020; 395:514–23. https://doi.org/10.1016/S0140-6736(20)30154-9 PMID:31986261

6. Phan LT, Nguyen TV, Luong QC, Nguyen TV, Nguyen HT, Le HQ, Nguyen TT, Cao TM, Pham QD. Importation and human-to-human transmission of a novel coronavirus in Vietnam. N Engl J Med. 2020; 382:872–74. https://doi.org/10.1056/NEJMc2001272 PMID:31991079

7. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. Gastroenterology. 2020; 158:1831–33.e3. https://doi.org/10.1053/j.gastro.2020.02.055 PMID:32142773

8. Tian S, Hu W, Niu L, Liu H, Xu H, Xiao SY. Pulmonary pathology of early-phase 2019 novel coronavirus (COVID-19) pneumonia in two patients with lung cancer. J Thorac Oncol. 2020; 15:700–04. https://doi.org/10.1016/j.jtho.2020.02.010 PMID:32114094

9. Yao XH, Li TY, He ZC, Ping YF, Liu HW, Yu SC, Mou HM, Wang LH, Zhang HR, Fu WJ, Luo T, Liu F, Guo QN, et al. [A pathological report of three COVID-19 cases by minimal invasive autopsies]. Zhonghua Bing Li Xue Za Zhi. 2020; 49:411–17. https://doi.org/10.3760/cma.j.cn112151-20200312-00193 PMID:32172546

10. Liang W, Feng Z, Rao S, Xiao C, Xue X, Lin Z, Zhang Q, Qi W. Diarrhoea may be underestimated: a missing link in 2019 novel coronavirus. Gut. 2020; 69:1141–43. https://doi.org/10.1136/gutjnl-2020-320832 PMID:32102928

11. Song Y, Liu P, Shi XL, Chu YL, Zhang J, Xia J, Gao XZ, Qu T, Wang MY. SARS-CoV-2 induced diarrhoea as onset symptom in patient with COVID-19. Gut. 2020; 69:1143–44. https://doi.org/10.1136/gutjnl-2020-320891 PMID:32139552

12. Mayumi T, Yoshida M, Tazuma S, Furukawa A, Nishii O, Shigematsu K, Azuhata T, Itakura A, Kamei S, Kondo H, Maeda S, Mihara H, Mizooka M, et al. The practice guidelines for primary care of acute abdomen 2015. Jpn J Radiol. 2016; 34:80–115. https://doi.org/10.1007/s11604-015-0489-z PMID:26678269

13. Gao Y, Xi H, Chen L. Emergency surgery in suspected COVID-19 patients with acute abdomen: case series and perspectives. Ann Surg. 2020; 272:e38–39. https://doi.org/10.1097/SLA.0000000000003961 PMID:32301807

14. Jamali S, Dagher M, Bilani N, Mailhac A, Habbal M, Zeineldine S, Tamim H. The effect of preoperative pneumonia on postsurgical mortality and morbidity: a NSQIP analysis. World J Surg. 2018; 42:2763–72. https://doi.org/10.1007/s00268-018-4539-4 PMID:29423742

15. Patterson JT, Bohl DD, Basques BA, Arzeno AH, Grauer JN. Does preoperative pneumonia affect complications of geriatric hip fracture surgery? Am J Orthop (Belle Mead NJ). 2017; 46:E177-85 PMID:28666049

16. Coccolini F, Perrone G, Chiarugi M, Di Marzo F, Ansaloni L, Scandroglio I, Marini P, Zago M, De Paolis P, Forfori F, Agresta F, Puzziello A, D'Ugo D, et al. Surgery in COVID-19 patients: operational directives. World J Emerg Surg. 2020; 15:25. https://doi.org/10.1186/s13017-020-00307-2 PMID:32264898

17. Aminian A, Safari S, Razeghian-Jahromi A, Ghorbani M, Delaney CP. COVID-19 outbreak and surgical practice: unexpected fatality in perioperative period. Ann Surg. 2020; 272:e27–29. https://doi.org/10.1097/SLA.0000000000003925 PMID:32221117

18. Mi B, Chen L, Xiong Y, Xue H, Zhou W, Liu G. Characteristics and early prognosis of COVID-19 infection in fracture patients. J Bone Joint Surg Am. 2020; 102:750–58. https://doi.org/10.2106/JBJS.20.00390 PMID:32379114

19. Blanco-Colino R, Vilallonga R, Martín R, Petrola C, Armengol M. Suspected acute abdomen as an extrapulmonary manifestation of covid-19 infection. Cir Esp. 2020; 98:295–96. https://doi.org/10.1016/j.ciresp.2020.03.006 PMID:32252978

20. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, Spitters C, Ericson K, Wilkerson S, Tural A, Diaz G, Cohn A, Fox L, et al, and Washington State 2019-nCoV Case Investigation Team. First case of 2019 novel coronavirus in the United States. N Engl J Med. 2020; 382:929–36. https://doi.org/10.1056/NEJMoa2001191 PMID:32004427

21. Harmer D, Gilbert M, Borman R, Clark KL. Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme. FEBS Lett. 2002; 532:107–10. https://doi.org/10.1016/s0014-5793(02)03640-2 PMID:12459472

22. Yan R, Zhang Y, Guo Y, Xia L, Zhou Q. Structural basis for the recognition of the 2019-nCoV by human ACE2. bioRxiv. 2020.

https://doi.org/10.1101/2020.02.19.956946

23. Tuech JJ, Gangloff A, Di Fiore F, Michel P, Brigand C, Slim K, Pocard M, Schwarz L. Strategy for the practice of digestive and oncological surgery during the covid-19 epidemic. J Visc Surg. 2020; 157:S7–12. https://doi.org/10.1016/j.jviscsurg.2020.03.008 PMID:32249098

24. Nibali L, Rizzo M, Li Volti G, D'Aiuto F, Giglio RV, Barbagallo I, Pelekos G, Donos N. Lipid subclasses profiles and oxidative stress in aggressive periodontitis before and after treatment. J Periodontal Res. 2015; 50:890–96. https://doi.org/10.1111/jre.12283 PMID:25994389

25. Kim R. Effects of surgery and anesthetic choice on immunosuppression and cancer recurrence. J Transl Med. 2018; 16:8. https://doi.org/10.1186/s12967-018-1389-7 PMID:29347949

26. Adhikari SP, Meng S, Wu YJ, Mao YP, Ye RX, Wang QZ, Sun C, Sylvia S, Rozelle S, Raat H, Zhou H. Epidemiology, causes, clinical manifestation and diagnosis, prevention and control of coronavirus disease (COVID-19) during the early outbreak period: a scoping review. Infect Dis Poverty. 2020; 9:29. https://doi.org/10.1186/s40249-020-00646-x PMID:32183901

Research Paper

Early coagulation tests predict risk stratification and prognosis of COVID-19

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ABSTRACT

The ongoing outbreak of Coronavirus Disease 2019 (COVID-19) is hitting the world hard, but the relationship between coagulation disorders and COVID-19 is still not clear. This study aimed to explore whether early coagulation tests can predict risk stratification and prognosis. PubMed, Web of Science, Cochrane Library, and Scopus were searched electronically for relevant research studies published up to March 24, 2020, producing 24 articles for the final inclusion. The pooled standard mean difference (SMD) of coagulation parameters at admission were calculated to determine severe and composite endpoint conditions (ICU or death) in COVID-19 patients. Meta-analyses revealed that platelet count was not statistically related to disease severity and composite endpoint; elevated D-dimer correlated positively with disease severity (SMD 0.787 (0.277-1.298), P= 0.003, I^2= 96.7%) but had no significant statistical relationship with composite endpoints. Similarly, patients with prolonged prothrombin time (PT) had an increased risk of ICU and increased risk of death (SMD 1.338 (0.551-2.125), P = 0.001, I^2 = 92.7%). Besides, increased fibrin degradation products (FDP) and decreased antithrombin might also mean the disease is worsening. Therefore, early coagulation tests followed by dynamic monitoring is useful for recognizing coagulation disorders accompanied by COVID-19 and guiding timely therapy to improve prognosis.

INTRODUCTION

In December 2019, a group of patients with unexplained pneumonia in Wuhan, China was found to be infected with a previously unknown coronavirus, officially named later as Coronavirus Disease 2019 (COVID-19). The coronavirus was initially called 2019-nCoV but was subsequently renamed severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) because it has 75-80% genomic similarity to SARS-CoV and 50% resemblance to the Middle East Respiratory Syndrome coronavirus (MERS-CoV) [1]. SARS-CoV2 is the third known kind of coronavirus that causes severe acute respiratory distress syndrome (ARDS) in humans, the others being SARS-CoV and MERS-CoV. As of April 7, 2020, 1,342,184 cases have been confirmed worldwide. Although the fatality rate will continue to change until all infected persons have recovered, it appears that SARS-CoV2 is less deadly (approximately 3.7%) than SARS-CoV (~10%) and much less than MERS-CoV (~40%) [2, 3]. Regrettably, the outbreak of COVID-19 is spreading wide and amplifying mainly because of the long incubation period and high infection rates, raising great public health concerns globally.

Unfortunately, some studies have revealed that mortality rates in critical COVID-19 patients are high (~41.7%), possibly because of the association of the disease with severe complications, including organ failure, sepsis/septic shock, and sepsis-associated coagulopathy [4–11]. Generally, the three conditions mentioned above are complexly linked in critical patients. Sepsis is consistently common in severe patients with SARS-CoV2 infection as a secondary disease [5]. Septic shock and sepsis-associated coagulopathy are severe conditions of sepsis, both of which can result in organ failure. The early reported incidence of at least one organ dysfunction is about 30%~60% in critically ill patients and non-survivors [5, 6, 12, 13], while the reported incidence of shock varies from 23% to 70% [5, 6, 13]. However, coagulopathy in COVID-19 has been reported rarely; only three articles have mentioned this problem up to now.

In the first report of the occurrence of disseminated intravascular coagulation (DIC), the worst form of coagulopathy, in a large epidemiological study on COVID-19, only 0.6% of the patients with severe cases had DIC; the standard used for diagnosis was not mentioned, and no one had DIC among non-severe patients [8]. Tang's analysis focusing on abnormal coagulation parameters revealed that 71.4% (15/21) of non-survivors with COVID-19 met the criteria for overt-DIC [11]. Zhou and his colleagues later found that 50% of non-survivors with COVID-19 had coagulopathy, and only 7% of survivors had coagulopathy [5]. However, DIC encompasses a broad spectrum of clinical manifestations, ranging from a prothrombotic state to bleeding or both [14], and there is a lack of a golden approach to diagnosing DIC, easily leading to misdiagnosis and missed diagnoses. To optimize patient care and resource allocation during this pandemic, coagulation parameters reflecting coagulopathy and DIC are urgently needed for risk stratification and for actively monitoring illness severity.

Abnormal coagulation parameters reflecting coagulopathy, including platelet count, D-dimer level, prothrombin time (PT), and activated partial thromboplastin time (APTT), are common in many COVID-19 patients at admission. However, these indicators, as presented in different articles, are providing contradictory messages to guiding risk stratification and predicting outcomes. Although two independent teams have shown that severe COVID-19 patients have significantly lower platelet counts than non-severe patients [10, 15], other teams have demonstrated that there is no significant difference between the two groups [6, 7, 13, 16–18]. Almost all related articles have reported that critical or non-survivor patients had statistically significantly higher levels of D-dimer than non-severe or survivor patients [4, 6, 10, 19–22], except for one [15]. PT is more prolonged in severe patients in some articles [6, 10, 11], but not so in other reports [4, 13, 19, 23]. APTT in severe COVID-19 patients appears more complicated, longer than in non-severe patients [10] or shorter than in non-severe patients [4, 21] or similar to the one in non-severe patients [6, 11, 13, 23, 24]. Some reports have shown that there is no significant difference in fibrinogen levels between severe COVID-19 patients and non-severe patients [11, 17, 19], but one article found higher levels in severe patients [23]. Therefore, we did a meta-analysis and a systematic review to comprehensively analyze the significance of early coagulation tests and understand coagulopathy during COVID-19 progression for disease stratification and prediction of the composite endpoint (ICU admission or death).

RESULTS

The outcome of the electronic search

Overall, 3370 documents were initially identified based on our search criteria and a reference list (Figure 1). Subsequently, 1669 files were excluded because of duplication, and 1627 were excluded after reading the title and abstract and finding that the materials were not related to medicine (n = 488) or failed to report clinical characteristics or laboratory tests (n = 657) or that they were reviews (n = 271), or expert consensus (n = 96), meta-analyses (n = 9), or case reports (n = 74). Additionally, 32 documents relating to children were excluded. As a result, 74 articles were selected for full-text assessment. Of the 74 studies, 50 were disqualified for lacking information on coagulation test data (n = 34), or having no definition of disease severity (n = 12), or lacking descriptive summary analyses (n = 3), or being a review (n = 1). In the end, 24 articles were included for the meta-analysis. To eliminate bias, the detailed endpoint was split into severity and composite endpoint instead of a rough poor outcome. Also, we analyzed several biomarkers individually rather than treat them as one entity.

Characteristics of the 24 selected studies

Of the articles included, 23 were full-length articles published in peer-reviewed journals, and one article was provided by the corresponding author after we reached out to them. Most of the studies were from China (n = 22), except for two from Singapore. All the investigations were case-control trials assessing 3544 adult COVID-19 patients; the sample size of each study varied from 21 to 1099 participants. The vast majority of patients were diagnosed using laboratory nucleic acid tests, except for three patients who were diagnosed based on clinical characteristics and imaging data. The details of the selected studies are provided in Table 1.

Ottawa quality assessment scale (NOS) was used to evaluate the quality of the chosen literature, and all literature scored ≥ 8 points (Supplementary Table 1), indicating that the quality of each of the 24 studies was high.

The relationship between platelets and disease severity or composite endpoint

The relationship between disease prognosis and platelets was analyzed in 16 articles with 2980 COVID-19 patients (Table 2). Of the 16 articles, 12 studies with 2152 patients were used to analyze the relationship between platelets and disease severity, [7, 8, 10, 12, 15, 17, 18, 22, 25–27] and 1778 patients in 6 articles were used to analyze the relationship between platelets and composite endpoint [6, 8, 12, 13, 16]. Pooled analyses revealed that platelet count was not statistically linked to disease severity (standard mean difference (SMD) -0.271 (-0.547-0.005), P = 0.054, I^2 = 84.6%) and composite endpoint (SMD -0.541 (-1.109-0.028), P = 0.062, I^2 = 92.5%) on admission (Table 2, Figures 2 and 3). Because the heterogeneity value was over 50%, the random effect model was used for the meta-analysis of these articles.

The relationship between D-dimer and disease severity or composite endpoint

In this meta-analysis, we explored the relationship between D-dimer and prognosis in 1762 patients with COVID-19 from 13 investigations (Table 2). Based on the data from 1438 participants in 11 trials, [4, 10, 15, 17, 19, 20, 22, 23, 25, 28].

We found that D-dimer correlated positively with disease severity in patients with COVID-19 (SMD 0.787 (0.277-1.298), P = 0.003, I^2 = 96.7%), suggesting that D-dimer levels were significantly elevated in critically ill patients. Also, 410 patients in three articles were assessed for the relationship between D-dimer and composite endpoint [5, 13], but we found no statistical relationship between the two parameters (SMD 1.523 (-0.221-3.267), P = 0.0087, I^2 = 97.5%), see Table 2, Figures 2 and 3.

The relationship between PT and disease severity or composite endpoint

Eleven articles with 1641 patients were analyzed for PT; 7 articles with 940 cases were evaluated for the relationship between PT and disease severity [4, 10, 19, 21, 23], and 5 articles with 645 cases were examined for the relationship between PT and composite endpoint [5, 11–13]. The analyses showed that prolonged PT during admission indicated a more serious disease, with the two correlating positively (SMD 0.803 (0.254-1.352), P = 0.004, I^2 = 91.3%). Similarly, patients with prolonged PT had an increased risk of ICU during admission and increased risk of death (SMD 1.338 (0.551-2.125), P = 0.001, I^2 = 92.7%), see Table 2, Figures 2 and 3.

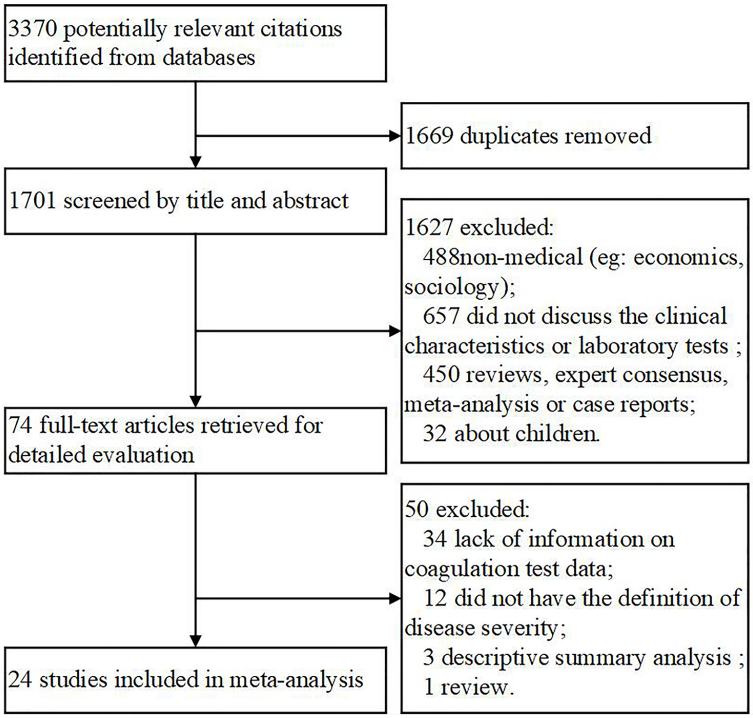

Figure 1. Flow chart of the included studies.

Table 1. Basic information of included studies.

Num	Study cohort	Journal	Institute/region	Period	Follow-up	Study type	No.(M/F)	Diagnose	Age (year)	Compared endpoint	NOS
1	Cao B [6]	Lancet	Jinyintan Hospital & Wuhan Pulmonary Hospital	2019/12/29-2020/1/31	NA	case control	191 (119/72)	laboratory-confirmed	56.0 (46.0–67.0)	composite endpoint	8
2	Sun ZY [11]	J. Thromb. Hemost.	Tongji Hospital	2020/1/1 - 2020/2/3	2020/2/13	case control	183 (98/85)	laboratory-confirmed	54.1 (14-94)	composite endpoint	8
3	Cao B (2) [5]	Lancet	Jinyintan Hospital	2019/12/16 -2020/1/2	2020/1/22	case control	41 (30/11)	laboratory-confirmed	49.0 (41·0-58.0)	composite endpoint	8
4	Ning Q [21]	NA	Tongji Hospital	2019/12/19-2020/1/27	2020/2/2	case control	21 (17/4)	laboratory-confirmed	56.3 (42.0-70.6)	severity status	8
5	Peng ZY [13]	JAMA	Zhongnan Hospital	2020/1/1-2020/1/28	2020/2/3	case control	138 (75/63)	laboratory-confirmed	56 (42-68)	composite endpoint	8
6	Zhong NS [8]	NA	552 hospitals	2020/1/29	2020/1/29	case control	1099 (640/459)	laboratory-confirmed	47.0 (35.0-58.0)	severity status/composite endpoint	8
7	Song YL [2]	JAMA Internal Medicine	Jinyintan Hospital	2019/12/24-2020/1/26	2020/2/13	case control	201 (128/73)	laboratory-confirmed	51(43-60)	severity status/composite endpoint	8
8	Hu B [22]	NA	Union Hospital	2020/1/16-2020/2/19	NA	case control	214 (127/87)	laboratory-confirmed	52.7 (37.2-68.2)	severity status	8
9	Zhang YX [15]	Clin Infect Dis	Zhongnan Hospital	2020/1/1-2020/2/5	NA	case control	155 (86/69)	laboratory-confirmed	54 (42-66)	severity status	8
10	Li LJ [36]	BMJ	Zhejiang Province	2020/1/10-2020/1/26	2020/1/26	case control	62 (36/27)	laboratory-confirmed	41 (32-52)	severity status	8
11	Shang Y [12]	The Lancet Respiratory Medicine	Jinyintan Hospital	2019/12-2020/1/26	2020/2/9	case control	52 (35/17)	laboratory-confirmed	59.7 (46.4-73.0)	composite endpoint	8
12	Ong, K H [16]	Am J Hematol	Singapore	2020/1/23-2020/2/28	2020/2/28	case control	67 (37/30)	laboratory-confirmed	42(35-54)	composite endpoint	8
13	Wang Q [18]	Journal of medical virology	Huizhou municipal central hospital from	2020/1-2020/2	2020/2/21	case control	30 (16/14)	laboratory-confirmed	50.5 (36-65)	severity status	8
14	Hu Y [27]	Chin Med J	Tongji Hospital	2019/12/30-2020/1/15	2019/12/30-2020/1/15	case control	78 (39/39)	laboratory-confirmed	38 (33-57)	severity status	8
15	Chen XM [17]	QJM	Zhejiang province	2020/1/20-2020/2/11	2020/2/16	case control	91 (37/54)	88 laboratory-confirmed & 3 clinical-confirmed	50 (36.5-57)	severity status	8
16	Gao YD [20]	Allergy	No. 7 Hospital of Wuhan	2020/1/16-2020/2/3	NA	case control	140 (71/69)	laboratory-confirmed	57 (25-87)	severity status	8
17	Zhang RG [7]	Clin Infect Dis	Union Hospital	2020/1/16-2020/1/29	2020/2/4	case control	69 (32/37)	laboratory-confirmed	42.0 (35.0-62.0)	severity status	8
18	Zhu CL [19]	Clinical chemistry and laboratory medicine	Renmin Hospital	2020/1/31-2020/2/10	NA	case control	134 (76/68)	laboratory-confirmed	NA	severity status	9
19	Wang LD [23]	Journal of medical virology	Fuyang Second people's hospital	2020/1/23-20202/2	NA	case control	43 (26/17)	laboratory-confirmed	43.74 ± 12.12	severity status	8
20	Zeng QT [24]	Zhonghua xin xue guan bing za zhi	Union Hospital	2020/1/20-2020/2/15	NA	case control	112 (53/59)	NA	62 (55-67)	severity status	8

21	Li CH [28]	Chinese journal of tuberculosis and respiratory diseases	Jianghan university hospital	2020/1/10-2020/1/31	NA	case control	30 (10/20)	laboratory-confirmed	35(27-43)	severity status	8
22	Barnaby EY [26]	JAMA	Singapore	2020/1/23-2020/2/3	2020/2/25	case control	18 (9/9)	laboratory-confirmed	47 (31-73)	severity status	8
23	Yang SR [10]	J Med Virol	Chongqing Three Gorges Central Hospital	2020/1/23-2020/2/8	NA	case control	135 (72/63)	laboratory-confirmed	47 (36-55)	severity status	8
24	Hu Y	NA	3 designated hospitals in Wuhan	2020/1/15-2020/2/15	2020/3/10	case control	380 (207/173)	laboratory-confirmed	64 (53-73)	severity status/composite endpoint	8

The second column is the corresponding author of the article. Composite endpoint means ICU or death. Not applicable (NA); M/F (male/female); Newcastle-Ottawa quality assessment scale (NOS).

Table 2. Summary of the meta-analysis results.

Biomarker	Total no. of studies	Total no. of patients	Endpoint	No. of studies	No. of patients	Statistical method	pooled Standard Mean Difference (SMD)	P	I^2	P (Heterogeneity)	P Begg's Test	P Egger's test
Platelet	16	2980	severity status	12	2152	I-V, Random	-0.271 (-0.547-0.005)	0.054	84.60%	<0.001	0.732	0.951
			composite endpoint	6	1778	I-V, Random	-0.541 (-1.109-0.028)	0.062	92.50%	<0.001	0.462	0.413
PT	11	1641	severity status	7	940	I-V, Random	0.803 (0.254-1.352)	**0.004**	91.30%	<0.001	0.368	0.224
			composite endpoint	5	645	I-V, Random	1.338 (0.551-2.125)	**0.001**	92.70%	<0.001	1.000	0.300
APTT	10	1388	severity status	7	940	I-V, Random	-0.133 (-0.668-0.402)	0.625	91.50%	<0.001	0.368	0.499
			composite endpoint	4	593	I-V, Random	0.327 (-0.630-1.285)	0.503	94.90%	<0.001	0.734	0.591
D-dimer	13	1762	severity status	11	1438	I-V, Random	0.787 (0.277-1.298)	**0.003**	96.70%	<0.001	0.062	0.510
			composite endpoint	3	410	I-V, Random	1.523 (-0.221-3.267)	0.087	97.50%	<0.001	1.000	0.805
Fibrinogen	5	682	-	-	-	I-V, Random	0.559 (-0.599-1.718)	0.344	96.70%	<0.001	0.806	0.317
FDP	3	548	-	-	-	I-V, Random	1.046 (0.371-1.722)	**0.002**	88.90%	<0.001	1.000	0.806
Antithrombin	3	548	-	-	-	I-V, Random	-0.798(-1.217 - -0.379)	**<0.001**	72.20%	0.027	0.296	0.190

prothrombin time (PT); activated partial thromboplastin time (APTT); fibrin/fibrinogen degradation products (FDP).

The relationship between APTT and disease severity or composite endpoint

10 articles with 1388 COVID-19 patients were analyzed for the relationship between disease prognosis and APTT (Table 2); 7 articles with 940 patients were assessed for the relationship between APTT and disease severity [4, 10, 19, 21, 23, 24], and 593 patients in four articles were studied for the relationship between APTT and composite endpoint [5, 11, 13]. Our results revealed that APTT was not statistically associated with disease severity and composite endpoint at admission (Table 2, Figures 2 and 3).

The relationship between fibrinogen, fibrin/ fibrinogen degradation products (FDP), anti-thrombin, and prognosis

Five studies with 682 patients were analyzed for the effect of fibrinogen on prognosis [11, 17, 19, 23]. We found that fibrinogen had no value in predicting disease prognosis in COVID-19 patients (SMD 0.559 (-0.599-1.718), P = 0.344, I^2 = 96.7%) (Supplementary Figure 1). Furthermore, 548 cases in three articles were evaluated for the relationship between FDP, antithrombin, and prognosis [11, 19]. Our results revealed that increased FDP (SMD 1.046 (0.371-1.722, P = 0.002, I^2 = 88.9%) and decreased antithrombin (SMD -0.798 (-1.217-0.379), P<0.001, I^2 = 72.2%) were associated with the worsening of COVID-19 (Table 2, Supplementary Figure 2).

Sensitivity analysis and publication bias

A Funnel plot was drawn to test publication bias, and Egger's test and Begg's test indicated that there was no publication bias (Supplementary Figures 3, 4). Sensitivity analysis revealed that no study greatly interfered with the results of this meta-analysis study greatly interfered with the results of this meta-analysis, suggesting that the study was stable (Supplementary Figures 5, 6).

DISCUSSION

COVID-19 has raised great public health concerns globally over the last three months. Like with SARS, abnormal coagulation disorders are common in severe patients with COVID-19. Our meta-analysis combined the outcomes of 3544 COVID-19 patients from 24 separate studies and established that elevated D-dimer significantly predicted more severe classifications of COVID-19 patients. Prolonged PT at baseline also suggested poor outcomes, both in severity status and composite endpoint. Increased FDPs and decreased antithrombin might also signal severe conditions.

The platelet count at admission had no remarkable relationship with outcome. However, a meta-analysis

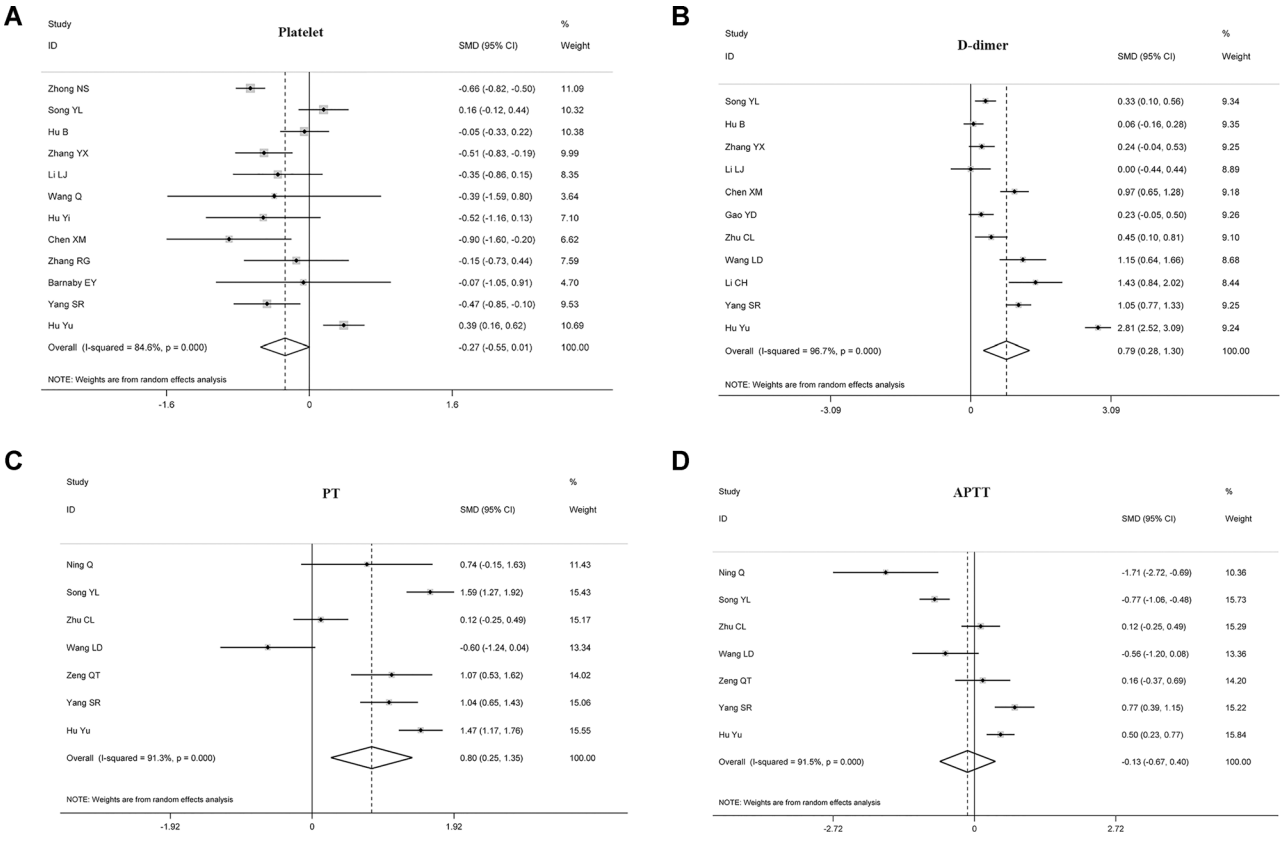

Figure 2. Forest plots assessing the severity status of COVID-19 patients, as determined using coagulation parameters. The sizes of the blocks or diamonds represent the weights, and the lengths of the straight lines represent the widths of the 95% CIs. (**A**) comparing patients by platelet counts; (**B**) comparing patients by D-dimer levels; (**C**) comparing patients by PT; (**D**) comparing patients by APTT. prothrombin time (PT); activated partial thromboplastin time (APTT).

involving 399 subjects showed that platelet counts at admission were significantly lower in more severe and non-survivor COVID-19 patients [29]. This discrepancy in outcome regarding platelet counts may be due to inconsistencies in the selected literature. A national multi-center retrospective study led by Academician Zhong supported the conclusion that platelet count is not statistically linked to a composite endpoint, although the authors also found that severe patients had lower platelets on admission than non-severe patients. One possible reason was the difference in the research objects. In other selected articles, the patients were either in Wuhan or outside Wuhan. The objects in Zhong's article included hospitalized patients both in Wuhan and outside-Wuhan. The early epidemic situation in Wuhan was overwhelming, medical resources were tight, and patients with a milder disease were isolated at home while more severe patients were admitted to the hospital. Patients hospitalized outside-Wuhan got sufficient resources due to they having relatively few cases at the time.

Platelets play a crucial role in hemostasis and thrombosis. While platelet activation and thrombocytosis increase the risk of thrombotic complications, platelet function disorders and thrombocytopenia increase bleeding risk. Thrombocytopenia and reactive thrombocytosis are both common in a variety of viral infections [30–35]. During SARS, most patients' platelet counts were normal at the onset of the disease, but, with time, 55% developed thrombocytopenia (platelet count $< 140\times10^9$/L), and 49% harbored reactive thrombocytosis (platelet count $\geq 400\times10^9$/L) [32]. Similarly, in COVID-19 patients, platelet counts were also within the normal range in most cases at admission [4, 7, 13, 15, 16, 26, 36]; thrombocytopenia (platelet count $< 100\times10^9$/L) was reported primarily in severe patients or non-survivors (20%~66.1%) [5, 8, 11], while thrombocytosis was reported in a few articles, and the proportion was not assessed [21]. The outcome of platelet count changes for the entirety of COVID-19 infection in patients has rarely been reported. Until recently, according to the article with 1476 COVID-19 patients by Yang et al., platelet counts in survivors tended to be stable during hospitalization, but they progressively decreased in non-survivors [37]. Furthermore, the lower the nadir platelet count during hospitalization, the higher the risk of death [37].

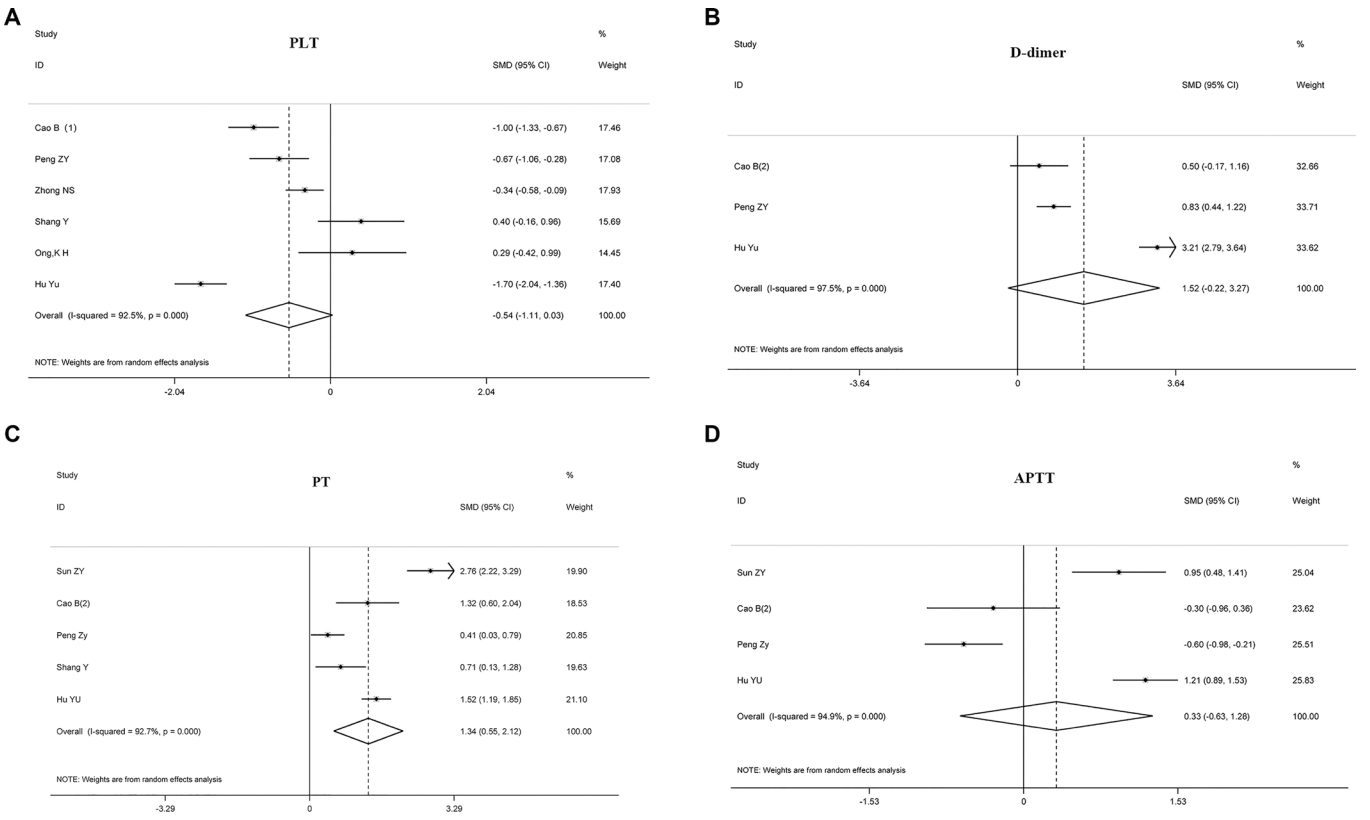

Figure 3. Forest plots assessing the composite endpoint of COVID-19 patients, as determined using coagulation parameters. The sizes of the blocks or diamonds represent the weights, and the lengths of the straight lines represent the widths of the 95% CIs. (**A**) Comparing patients by platelet counts; (**B**) comparing patients by D-dimer levels; (**C**) comparing patients by PT; (**D**) comparing patients by APTT. prothrombin time (PT); activated partial thromboplastin time (APTT).

Thrombocytopenia is often considered an indicator of bleeding and mortality in critical patients [38]. Decreased platelet counts help recognize the presence and severity of coagulopathy [39]. The mechanisms of thrombocytopenia during COVID-19 might include direct or indirect factors induced by the SARS-Cov2 infection, such as inappropriate platelet activation and consumption, immunological platelet destruction, and impaired megakaryopoiesis [40]. Recently, Levi M et al. proposed that localized pulmonary thrombotic microangiopathy where platelet consumption is a common feature, may partly account for thrombocytopenia [41]. Additionally, two independent teams found that COVID-19 patients in ICU had markedly elevated levels of the von Willebrand factor [42, 43], further supporting Levi M's opinion. Though COVID-19-associated coagulopathy belongs to sepsis-induced coagulopathy, thrombocytopenia is less profound [43], which may be related with that COVID-19-accociated-coagulopathy was a severe hypercoagulability rather than consumptive coagulopathy [44]. Bleeding events are less documented or reported in current articles looking at the clinical features of COVID-19, although autopsies have revealed focal hemorrhage in the lungs and spleen and decreased myelopoiesis in the bone marrow [45]. Mao's team found that one of 88 severe patients had a cerebral hemorrhage [21]. Yang et al. showed that 6% of 32 non-survivors had a gastrointestinal hemorrhage [12]. In addition to low platelets, bleeding events in critical COVID-19 patients may also be linked to corticosteroid therapy in more critically ill patients. In the interim guidance of coagulopathy in COVID-19, the ISTH recommends that platelet counts be kept above 50×10^9/L in bleeding patients and above 20×10^9/L in non-bleeding patients.

D-dimer, a more specific marker than FDP reflecting the dissolution of microthrombi, is amplified in septic patients [46], consistent with what is reported in COVID-19 patients [6, 10, 11, 20, 22, 23]. In non-COVID-19 septic patients, D-dimer concentrations do not reach the high values seen in patients with COVID-19 [41, 43]. Generally, FDP correlates positively well with D-dimer, except in some situations, like primary hyperfibrinolysis, and simultaneous measurements of FDPs and D-dimer are useful for more accurate estimations of fibrinolytic states [47]. However, of the articles that met our inclusion criteria, only three provided FDP information, whereas, many articles recorded D-dimer changes. Strikingly, 43.2%~68% of COVID-19 patients had elevated levels of D-dimer [5, 8, 20], and this proportion was as high as 92% in dead patients [5]. Increased D-dimer levels generally indicate a high risk of thrombotic diseases [48]. By the time we started this meta-analysis, the incidence of thrombosis had rarely been reported in COVID-19 patients, although thrombosis and microthrombosis in multiple organs had been observed during autopsies [45]. In a study specifically looking at neurological manifestations, Mao and colleagues revealed that 4.5% of severe COVID-19 patients had an acute ischemic stroke [22]. Another study found that 3.4% of severe COVID-19 patients had a stroke [20]. Recently, several teams in different countries emphasized the high incidence of thrombotic events in severe COVID-19 patients. In a study of 81 ICU patients without routine thromboprophylaxis in China, the incidence of deep vein thrombosis was 25% [49]. In Netherlands, two independent researches where routine low molecular weight heparin prophylaxis was applied, reported similar (even higher) incidence of venous thromboembolism (VTE) among ICU patients with COVID-19 [50, 51]. Most recently, Helms et al. showed that COVID-19-ARDS patients developed significantly more thrombotic complications than non-COVID-19-ARDS patients based on a multicenter prospective cohort study [43].

In COVID-19 patients, especially severe patients, the mechanisms of elevated D-dimer or thrombosis may include older age, chronic diseases, hypoxemia, hypercytokinemia, coagulopathy, and inevitable prolonged bed rest. It is already well-established that older individuals and those who have co-morbidities and hypercytokinemia are more likely to die from COVID-19 infection [4, 5, 7, 8, 11, 20, 21, 23, 24]. Aging and chronic diseases are recognized risk factors for sepsis, which is characterized by excessive inflammation, including hypercytokinemia and endothelial dysfunction, resulting in a hypercoagulability state [42, 52]. Refractory hypoxemia may lead to vasoconstriction reducing blood flow and promoting vascular occlusion [53]. SARS- and COVID-19-associated coagulopathy is sepsis-induced, generally characterized by markedly increased levels of plasminogen activator inhibitor-1 (PAI-1) [46, 54]. Consistently, the PAI-1 level in SARS patients is significantly higher, not only compared to healthy controls but also patients with other cases of pneumonia [55], and whether this is so in COVID-19 patients is a matter still to be verified.

Generally, coagulation tests are prolonged when the level of coagulation factors is below 50%, and an abnormality may occur up to the decompensation period of DIC because of the consumption of clotting factors during DIC progression [46, 56]. However, at the early stage of septic DIC, coagulation tests may be shortened because of hypercoagulability. This meta-analysis showed that PT, but not APTT, had an increased risk of ICU and death on admission, perhaps because coagulopathy in COVID-19 is sepsis-induced, where mostly the exogenous, but not the endogenous,

coagulation pathway is activated. Given that PT and APTT are within the reference ranges on admission in most COVID-19 patients, baseline PT and APTT have limited values for risk stratification and prognosis in COVID-19 patients [5, 13, 19]. However, PT can progressively extend in nonsurvivors [11], due to the continuous activation and consumption of the exogenous coagulation pathway. As an acute reactive protein, hyperfibrinogenemia is common in the early phase of COVID-19 in both survivors and non-survivors [11]. Yet, the level of fibrinogen can progressively decrease in non-survivors, and hypo-fibrinogenemia may be observed at the late stage of consumption coagulopathy [11]. Antithrombin may be readily exhausted during continuous thrombin generation, with low levels of antithrombin found in approximately 50% of critically ill patients and 90% of DIC patients [56]. Therefore, the dynamic monitoring of these coagulation tests is highly recommended.

The combination of thrombocytopenia, increased D-dimer, prolonged PT, and decreased antithrombin is suggestive of DIC, though the majority of COVID-19 patients would not meet the Overt-DIC criteria established by the International Society on Thrombosis and Hemostasis (ISTH) [41, 43]. The ISTH positively recommends anticoagulants when septic patients meet the diagnostic criteria of sepsis-induced coagulopathy (SIC) [54], which could result in a significant reduction in mortality [57, 58]. However, patients with advanced coagulopathy may have a disease progression that is no longer amenable to anticoagulant therapy [59]. For that reason, the ISTH recommends a two-step diagnosis for sepsis-associated coagulopathy and emphasizes that therapeutic doses of heparin should be considered in coagulopathic patients to avoid progression from coagulopathy to DIC [54]. Increasing evidence demonstrates that there is a high risk of thrombotic complications in severe COVID-19 patients, and early anticoagulation therapy seems to improve the outcome of severe COVID-19 patients [43, 49–51, 60–62]. Tang's team specifically looking at anticoagulant treatments showed that the 28-day mortality rate of COVID-19 patients using heparin was lower than that of nonusers in cases of severe COVID-19 patients meeting SIC criteria or with D-dimer > 3.0 ug/mL [60]. Llitjos et al. revealed that, among the twenty-six COVID-19 patients with mechanical ventilation, the incidence of VTE in patients treated with prophylactic anticoagulation was significantly higher than that in the group receiving therapeutic anticoagulation [63]. In a prospective observational study with sixteen ICU COVID-19 patients, Ranucci et al. showed that the pro-coagulant situation of patients gradually improved after thromboprophylaxis was increased [64]. Zhang et al. revealed that the thromboprophylaxis halved the incidence of DVT in COVID-19 patients with a Padua prediction score≥4 [65]. Given that COVID-19-ARDS patients had higher risk of thrombotic complications than non-COVID-19-ARDS patients, Helms et al. suggested the presence of higher anticoagulation targets in critically ill patients than usual [43]. However, the efficacy of anticoagulant therapy needs to be verified in high-quality RCT experiments. Clinicians should closely monitor indicators during the laboratory examination of patients to stay alert for side effects after anticoagulant treatment [66].

Our study has several limitations. First, all the studies included in this meta-analysis are retrospective studies with large heterogeneity. Second, the data came mainly from China; factors such as virus strain types, medical levels, countries, races, etc., may affect the results. However, at the moment, more detailed subgroup analyses cannot be conducted to comprehensively understand COVID-19 because the material for this is limited. Third, for some parameters, the number of studies included in the meta-analysis was less than 10. In this case, the publication bias may, therefore, not have been detected by Egger's and Begg's tests because of the relatively lower power. Fourth, the pooled sample sizes were not large enough. Precise estimates of these parameters should be assessed further.

CONCLUSIONS

Elevated D-dimer and FDP, prolonged PT, and decreased antithrombin predict higher risk stratification and poorer prognosis in COVID-19, which is perhaps not fully in line with the facts because the studies selected for the meta-analysis were limited. There is, however, no doubt that early coagulation tests and dynamically monitoring coagulation indicators during hospitalization are helpful in the early identification of coagulation disorders, and the rational use of these parameters and the scoring systems help guide treatment and improve the prognosis of COVID-19.

MATERIALS AND METHODS

Search strategy

We conducted this systematic review and meta-analysis using a predefined protocol under PRISMA guidelines [67]. We searched PubMed, Web of Science, Cochrane Library, and Scopus electronically. Medical subject headings and random words (e.g. COVID-19) were combined to search the databases without language or ethnic origin restriction and dated up to March 24, 2020 (for detailed search methods, see Supplementary Table 2). The titles, abstracts, and full texts of all documents were identified independently by two investigators, and disagreements were adjudicated by a third investigator.

The reference list of all identified documents was scrutinized to identify additional potentially eligible studies.

Inclusion and exclusion

The criteria for including a study in the meta-analysis were as follows: (I) the COVID-19 patient cohort was confirmed primarily by laboratory detection; (II) the endpoint was severity status and/or composite endpoint (including ICU monitoring and death); (III) groups were established for comparison; (IV) the correlation of coagulation biomarkers with endpoints was recorded. Exclusion criteria were as follows: (I) review articles, case reports, and laboratory studies; (II) studies with insufficient data for estimating pooled standard mean differences (SMD).

Data extraction

We collected the following items from each study, if available: the corresponding author's name, the study type, the institute or region, the period of case collection and follow-up, the number of reported cases, disease severity, complications (e.g., coagulopathy and DIC), outcome, and laboratory findings (e.g., platelet, D-dimer, PT, APTT, or fibrinogen) were entered in a well-designed form independently by two investigators. If different articles published by the same institution overlapped during case inclusion, the research with the largest number of cases was selected, and the others were excluded. A third investigator checked the article list and data extraction to ensure that there were no duplicate articles or duplicate information and made a judgment on controversial articles.

Quality assessment

Two reviewers independently evaluated the methodological quality of each selected study. The quality of case-control studies was evaluated using the Newcastle-Ottawa quality assessment scale (NOS) [68], which comprises 9 points; 4 points for selection, 2 points for comparability, and 3 points for the outcome. Six or more points in case-control studies were regarded as high quality. Disagreements were resolved by discussion.

Statistical analysis

Version 12.0 of the STATA statistical software (STATA, College Station, TX) was used to calculate the combined survival impact of indicators of coagulation. The impact of biomarkers on endpoints was determined by calculating pooled mean values and their 95% CIs. Results suggested statistical significance if the 95% CI was no more than 0. Also, increased indicator levels contributed to an adverse survival effect, compared to control-patients, when the pooled mean value was more than 0. The heterogeneity of the selected studies was evaluated using the chi-squared test, with significance set at a p-value of less than 0.10. The statistic I^2 was used to quantify heterogeneity; an I^2 value less than 25% was regarded as low heterogeneity, a value between 25 and 50% indicated moderate heterogeneity, and a value over 50% signaled high heterogeneity [69]. The random-effect model was used if high heterogeneity was observed; otherwise, a fixed-effect model was used for the meta-analysis. Sensitivity analysis was applied to explore the origin of heterogeneity. Funnel plots, Begg's test, and Egger's test were used to screen for potential publication bias of the total population. Poor stability resulting from the inclusion and exclusion of studies was reappraised.

Abbreviations

COVID-19: Coronavirus Disease 2019; SARS-Cov-2: severe acute respiratory syndrome coronavirus 2; ARDS: acute respiratory distress syndrome; MERS-CoV: Middle East Respiratory Syndrome coronavirus; DIC: disseminated intravascular coagulation; PT: prothrombin time; APTT: activated partial thromboplastin time; FDP: fibrin/fibrinogen degradation products; SMD: standard mean differences; VTE: venous thromboembolism. NOS:Newcastle-Ottawa quality assessment scale.

AUTHOR CONTRIBUTIONS

MH and HY conceived and designed the study. LLL, XM, DMY, KHM, LDY and CZP contributed to the literature searches, study selection, data extraction, quality assessment, data analysis and interpretation. LLL, XM and DMY drafted the initial manuscript, and MH and HY made critical revisions to the intellectual content. All authors approved the final version of the study.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest

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REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020; 382:727–733. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

2. Yang S, Wu J, Ding C, Cui Y, Zhou Y, Li Y, Deng M, Wang C, Xu K, Ren J, Ruan B, Li L. Epidemiological features of and changes in incidence of infectious diseases in China in the first decade after the SARS outbreak: an observational trend study. Lancet Infect Dis. 2017; 17:716–25. https://doi.org/10.1016/S1473-3099(17)30227-X PMID:28412150

3. Chen J. Pathogenicity and transmissibility of 2019-nCoV-a quick overview and comparison with other emerging viruses. Microbes Infect. 2020; 22:69–71. https://doi.org/10.1016/j.micinf.2020.01.004 PMID:32032682

4. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Zhou X, Du C, Zhang Y, Song J, Wang S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern Med. 2020; 180:934. https://doi.org/10.1001/jamainternmed.2020.0994 PMID:32167524

5. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

6. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

7. Wang Z, Yang B, Li Q, Wen L, Zhang R. Clinical features of 69 cases with coronavirus disease 2019 in Wuhan, China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa272 PMID:32176772

8. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

9. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

10. Wan S, Xiang Y, Fang W, Zheng Y, Li B, Hu Y, Lang C, Huang D, Sun Q, Xiong Y, Huang X, Lv J, Luo Y, et al. Clinical features and treatment of COVID-19 patients in northeast Chongqing. J Med Virol. 2020; 92:797–806. https://doi.org/10.1002/jmv.25783 PMID:32198776

11. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. J Thromb Haemost. 2020; 18:844–847. https://doi.org/10.1111/jth.14768 PMID:32073213

12. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–481. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

13. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

14. Ralph AG, Brainard BM. Update on disseminated intravascular coagulation: when to consider it, when to expect it, when to treat it. Top Companion Anim Med. 2012; 27:65–72. https://doi.org/10.1053/j.tcam.2012.06.004 PMID:23031458

15. Mo P, Xing Y, Xiao Y, Deng L, Zhao Q, Wang H, Xiong Y, Cheng Z, Gao S, Liang K, Luo M, Chen T, Song S, et al. Clinical characteristics of refractory COVID-19 pneumonia in Wuhan, China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa270 PMID:32173725

16. Fan BE, Chong VC, Chan SS, Lim GH, Lim KG, Tan GB, Mucheli SS, Kuperan P, Ong KH. Hematologic parameters in patients with COVID-19 infection. Am J Hematol. 2020; 95:E131–34. https://doi.org/10.1002/ajh.25774 PMID:32129508

17. Qian GQ, Yang NB, Ding F, Ma AH, Wang ZY, Shen YF, Shi CW, Lian X, Chu JG, Chen L, Wang ZY, Ren DW, Li GX, et al. Epidemiologic and clinical characteristics of 91 hospitalized patients with COVID-19 in Zhejiang, China: a retrospective, multi-centre case series. QJM. 2020. 113:474–81 https://doi.org/10.1093/qjmed/hcaa089 PMID:32181807

18. Qu R, Ling Y, Zhang YH, Wei LY, Chen X, Li XM, Liu XY, Liu HM, Guo Z, Ren H, Wang Q. Platelet-to-lymphocyte ratio is associated with prognosis in patients with coronavirus disease-19. J Med Virol. 2020; 10:1002. https://doi.org/10.1002/jmv.25767 PMID:32181903

19. Han H, Yang L, Liu R, Liu F, Wu KL, Li J, Liu XH, Zhu CL. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. Clin Chem Lab Med. 2020; 58:1116–20. https://doi.org/10.1515/cclm-2020-0188 PMID:32172226

20. Zhang JJ, Dong X, Cao YY, Yuan YD, Yang YB, Yan YQ, Akdis CA, Gao YD. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. Allergy. 2020. 75:1730–41. https://doi.org/10.1111/all.14238 PMID:32077115

21. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang X, Zhang M, Wu S, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest. 2020; 130:2620–29. https://doi.org/10.1172/JCI137244 PMID:32217835

22. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, Chang J, Hong C, Zhou Y, Wang D, Miao X, Li Y, Hu B. Neurologic Manifestations of Hospitalized Patients With Coronavirus Disease 2019 in Wuhan, China. JAMA Neurol. 2020; 77:1–9. [Epub ahead of print]. https://doi.org/10.1001/jamaneurol.2020.1127 PMID:32275288

23. Gao Y, Li T, Han M, Li X, Wu D, Xu Y, Zhu Y, Liu Y, Wang X, Wang L. Diagnostic utility of clinical laboratory data determinations for patients with the severe COVID-19. J Med Virol. 2020; 92:791–96. https://doi.org/10.1002/jmv.25770 PMID:32181911

24. Peng YD, Meng K, Guan HQ, Leng L, Zhu RR, Wang BY, He MA, Cheng LX, Huang K, Zeng QT. [Clinical characteristics and outcomes of 112 cardiovascular disease patients infected by 2019-nCoV]. Zhonghua Xin Xue Guan Bing Za Zhi. 2020; 48:450-55. https://doi.org/10.3760/cma.j.cn112148-20200220-00105 PMID:32120458

25. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, Li SB, Wang HY, Zhang S, Gao HN, Sheng JF, Cai HL, Qiu YQ, Li LJ. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series. BMJ. 2020; 368:m606. https://doi.org/10.1136/bmj.m606 PMID:32075786

26. Young BE, Ong SW, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, et al, and Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. JAMA. 2020; 323:1488–94. https://doi.org/10.1001/jama.2020.3204 PMID:32125362

27. Liu W, Tao ZW, Wang L, Yuan ML, Liu K, Zhou L, Wei S, Deng Y, Liu J, Liu HG, Yang M, Hu Y. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. Chin Med J (Engl). 2020; 133:1032–38. https://doi.org/10.1097/CM9.0000000000000775 PMID:32118640

28. Liu M, He P, Liu HG, Wang XJ, Li FJ, Chen S, Lin J, Chen P, Liu JH, Li CH. [Clinical characteristics of 30 medical workers infected with new coronavirus pneumonia]. Zhonghua Jie He He Hu Xi Za Zhi. 2020; 43:209–214. https://doi.org/10.3760/cma.j.issn.1001-0939.2020.03.014 PMID:32164090

29. Lippi G, Plebani M, Henry BM. Thrombocytopenia is associated with severe coronavirus disease 2019 (COVID-19) infections: a meta-analysis. Clin Chim Acta. 2020; 506:145–48. https://doi.org/10.1016/j.cca.2020.03.022 PMID:32178975

30. Ballem PJ, Belzberg A, Devine DV, Lyster D, Spruston B, Chambers H, Doubroff P, Mikulash K. Kinetic studies of the mechanism of thrombocytopenia in patients with human immunodeficiency virus infection. N Engl J Med. 1992; 327:1779–84. https://doi.org/10.1056/NEJM199212173272503 PMID:1435932

31. Flaujac C, Boukour S, Cramer-Bordé E. Platelets and viruses: an ambivalent relationship. Cell Mol Life Sci. 2010; 67:545–56. https://doi.org/10.1007/s00018-009-0209-x PMID:20012669

32. Wong RS, Wu A, To KF, Lee N, Lam CW, Wong CK, Chan PK, Ng MH, Yu LM, Hui DS, Tam JS, Cheng G, Sung JJ. Haematological manifestations in patients with severe acute respiratory syndrome: retrospective analysis. BMJ. 2003; 326:1358–62. https://doi.org/10.1136/bmj.326.7403.1358 PMID:12816821

33. Assinger A. Platelets and infection - an emerging role of platelets in viral infection. Front Immunol. 2014; 5:649. https://doi.org/10.3389/fimmu.2014.00649 PMID:25566260

34. Schafer AI. Thrombocytosis. JAMA. 2015; 314:1171–72. https://doi.org/10.1001/jama.2015.8515 PMID:26372588

35. Harrison CN, Bareford D, Butt N, Campbell P, Conneally E, Drummond M, Erber W, Everington T, Green AR, Hall GW, Hunt BJ, Ludlam CA, Murrin R, et al, and British Committee for Standards in Haematology. Guideline for investigation and management of adults and children presenting with a thrombocytosis. Br J Haematol. 2010; 149:352–75. https://doi.org/10.1111/j.1365-2141.2010.08122.x PMID:20331456

36. Chen R, Sang L, Jiang M, Yang Z, Jia N, Fu W, Xie J, Guan W, Liang W, Ni Z, Hu Y, Liu L, Shan H, et al. Longitudinal hematologic and immunologic variations associated with the progression of COVID-19 patients in China. J Allergy Clin Immunol. 2020; 146:89–100. https://doi.org/10.1016/j.jaci.2020.05.003 PMID:32407836

37. Yang X, Yang Q, Wang Y, Wu Y, Xu J, Yu Y, Shang Y. Thrombocytopenia and its association with mortality in patients with COVID-19. J Thromb Haemost. 2020; 18:1469–72. https://doi.org/10.1111/jth.14848 PMID:32302435

38. Williamson DR, Albert M, Heels-Ansdell D, Arnold DM, Lauzier F, Zarychanski R, Crowther M, Warkentin TE, Dodek P, Cade J, Lesur O, Lim W, Fowler R, et al, and PROTECT collaborators, the Canadian Critical Care Trials Group, the Australian and New Zealand Intensive Care Society Clinical Trials Group. Thrombocytopenia in critically ill patients receiving thromboprophylaxis: frequency, risk factors, and outcomes. Chest. 2013; 144:1207–15. https://doi.org/10.1378/chest.13-0121 PMID:23788287

39. Taylor FB Jr, Toh CH, Hoots WK, Wada H, Levi M, and Scientific Subcommittee on Disseminated Intravascular Coagulation (DIC) of the International Society on Thrombosis and Haemostasis (ISTH). Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. Thromb Haemost. 2001; 86:1327–30. PMID:11816725

40. Amgalan A, Othman M. Exploring possible mechanisms for COVID-19 induced thrombocytopenia: unanswered questions. J Thromb Haemost. 2020; 18:1514–16. https://doi.org/10.1111/jth.14832 PMID:32278338

41. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. Lancet Haematol. 2020; 7:e438–40. https://doi.org/10.1016/S2352-3026(20)30145-9 PMID:32407672

42. Panigada M, Bottino N, Tagliabue P, Grasselli G, Novembrino C, Chantarangkul V, Pesenti A, Peyvandi F, Tripodi A. Hypercoagulability of COVID-19 patients in intensive care unit. A report of thromboelastography findings and other parameters of hemostasis. J Thromb Haemost. 2020; 18:1738–42. https://doi.org/10.1111/jth.14850 PMID:32302438

43. Helms J, Tacquard C, Severac F, Leonard-Lorant I, Ohana M, Delabranche X, Merdji H, Clere-Jehl R, Schenck M, Fagot Gandet F, Fafi-Kremer S, Castelain V, Schneider F, et al, and CRICS TRIGGERSEP Group (Clinical Research in Intensive Care and Sepsis Trial Group for Global Evaluation and Research in Sepsis). High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. Intensive Care Med. 2020; 46:1089–98. https://doi.org/10.1007/s00134-020-06062-x PMID:32367170

44. Spiezia L, Boscolo A, Poletto F, Cerruti L, Tiberio I, Campello E, Navalesi P, Simioni P. COVID-19-related severe hypercoagulability in patients admitted to intensive care unit for acute respiratory failure. Thromb Haemost. 2020; 120:998–1000. https://doi.org/10.1055/s-0040-1710018 PMID:32316063

45. National Health Commission & National Administration of Traditional Chinese Medicine. Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7). Chin Med J (Engl). 2020; 133:1087–1095. https://doi.org/10.1097/CM9.0000000000000819 PMID:32358325

46. Asakura H. Classifying types of disseminated intravascular coagulation: clinical and animal models. J Intensive Care. 2014; 2:20. https://doi.org/10.1186/2052-0492-2-20 PMID:25520834

47. Sato N, Takahashi H, Shibata A. Fibrinogen/fibrin degradation products and d-dimer in clinical practice: interpretation of discrepant results. Am J Hematol. 1995; 48:168–74. https://doi.org/10.1002/ajh.2830480306 PMID:7864025

48. Johnson ED, Schell JC, Rodgers GM. The d-dimer assay. Am J Hematol. 2019; 94:833–39. https://doi.org/10.1002/ajh.25482

PMID:30945756

49. Cui S, Chen S, Li X, Liu S, Wang F. Prevalence of venous thromboembolism in patients with severe novel coronavirus pneumonia. J Thromb Haemost. 2020; 18:1421–24. https://doi.org/10.1111/jth.14830 PMID:32271988

50. Klok FA, Kruip MJ, van der Meer NJ, Arbous MS, Gommers D, Kant KM, Kaptein FH, van Paassen J, Stals MA, Huisman MV, Endeman H. Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: an updated analysis. Thromb Res. 2020; 191:148–50. https://doi.org/10.1016/j.thromres.2020.04.041 PMID:32381264

51. Middeldorp S, Coppens M, van Haaps TF, Foppen M, Vlaar AP, Müller MC, Bouman CC, Beenen LF, Kootte RS, Heijmans J, Smits LP, Bonta PI, van Es N. Incidence of venous thromboembolism in hospitalized patients with COVID-19. J Thromb Haemost. 2020; 18:1995-2002. https://doi.org/10.1111/jth.14888 PMID:32369666

52. Bermejo-Martin JF, Martín-Fernandez M, López-Mestanza C, Duque P, Almansa R. Shared Features of Endothelial Dysfunction between Sepsis and Its Preceding Risk Factors (Aging and Chronic Disease). J Clin Med. 2018; 7:400. https://doi.org/10.3390/jcm7110400 PMID:30380785

53. Grimmer B, Kuebler WM. The endothelium in hypoxic pulmonary vasoconstriction. J Appl Physiol (1985). 2017; 123:1635–46. https://doi.org/10.1152/japplphysiol.00120.2017 PMID:28860164

54. Iba T, Levy JH, Warkentin TE, Thachil J, van der Poll T, Levi M, and Scientific and Standardization Committee on DIC, and the Scientific and Standardization Committee on Perioperative and Critical Care of the International Society on Thrombosis and Haemostasis. Diagnosis and management of sepsis-induced coagulopathy and disseminated intravascular coagulation. J Thromb Haemost. 2019; 17:1989–1994. https://doi.org/10.1111/jth.14578 PMID:31410983

55. Wu YP, Wei R, Liu ZH, Chen B, Lisman T, Ren DL, Han JJ, Xia ZL, Zhang FS, Xu WB, Preissner KT, de Groot PG. Analysis of thrombotic factors in severe acute respiratory syndrome (SARS) patients. Thromb Haemost. 2006; 96:100–1. https://doi.org/10.1160/TH05-12-0827 PMID:16807662

56. Levi M, Meijers JC. DIC: which laboratory tests are most useful. Blood Rev. 2011; 25:33–37. https://doi.org/10.1016/j.blre.2010.09.002 PMID:20950905

57. Kienast J, Juers M, Wiedermann CJ, Hoffmann JN, Ostermann H, Strauss R, Keinecke HO, Warren BL, Opal SM, and KyberSept investigators. Treatment effects of high-dose antithrombin without concomitant heparin in patients with severe sepsis with or without disseminated intravascular coagulation. J Thromb Haemost. 2006; 4:90–7. https://doi.org/10.1111/j.1538-7836.2005.01697.x PMID:16409457

58. Dhainaut JF, Yan SB, Joyce DE, Pettilä V, Basson B, Brandt JT, Sundin DP, Levi M. Treatment effects of drotrecogin alfa (activated) in patients with severe sepsis with or without overt disseminated intravascular coagulation. J Thromb Haemost. 2004; 2:1924–33. https://doi.org/10.1111/j.1538-7836.2004.00955.x PMID:15550023

59. Gando S, Iba T, Eguchi Y, Ohtomo Y, Okamoto K, Koseki K, Mayumi T, Murata A, Ikeda T, Ishikura H, Ueyama M, Ogura H, Kushimoto S, et al; and Japanese Association for Acute Medicine Disseminated Intravascular Coagulation (JAAM DIC) Study Group. A multicenter, prospective validation of disseminated intravascular coagulation diagnostic criteria for critically ill patients: comparing current criteria. Crit Care Med. 2006; 34:625–31. https://doi.org/10.1097/01.ccm.0000202209.42491.38 PMID:16521260

60. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. J Thromb Haemost. 2020; 18:1094–1099. https://doi.org/10.1111/jth.14817 PMID:32220112

61. Lodigiani C, Iapichino G, Carenzo L, Cecconi M, Ferrazzi P, Sebastian T, Kucher N, Studt JD, Sacco C, Alexia B, Sandri MT, Barco S; Humanitas COVID-19 Task Force. Venous and arterial thromboembolic complications in COVID-19 patients admitted to an academic hospital in Milan, Italy. Thromb Res. 2020; 191:9–14. https://doi.org/10.1016/j.thromres.2020.04.024 PMID:32353746

62. Llamas-Velasco M, Muñoz-Hernández P, Lázaro-González J, Reolid-Pérez A, Abad-Santamaría B, Fraga J, Daudén-Tello E. Thrombotic occlusive vasculopathy in skin biopsy from a livedoid lesion of a patient with COVID-19. Br J Dermatol. 2020; 10:1111. https://doi.org/10.1111/bjd.19222 PMID:32407552

63. Llitjos JF, Leclerc M, Chochois C, Monsallier JM, Ramakers M, Auvray M, Merouani K. High incidence of venous thromboembolic events in anticoagulated severe COVID-19 patients. J Thromb Haemost. 2020; 18:1743–46. https://doi.org/10.1111/jth.14869 PMID:32320517

64. Ranucci M, Ballotta A, Di Dedda U, Bayshnikova E, Dei Poli M, Resta M, Falco M, Albano G, Menicanti L. The procoagulant pattern of patients with COVID-19 acute respiratory distress syndrome. J Thromb Haemost. 2020. 18:1747–51. https://doi.org/10.1111/jth.14854 PMID:32302448

65. Zhang L, Feng X, Zhang D, Jiang C, Mei H, Wang J, Zhang C, Li H, Xia X, Kong S, Liao J, Jia H, Pang X, et al. Deep vein thrombosis in hospitalized patients with coronavirus disease 2019 (COVID-19) in Wuhan, China: prevalence, risk factors, and outcome. Circulation. 2020. [Epub ahead of print]. https://doi.org/10.1161/CIRCULATIONAHA.120.046702 PMID:32421381

66. Lin L, Lu L, Cao W, Li T. Hypothesis for potential pathogenesis of SARS-CoV-2 infection-a review of immune changes in patients with viral pneumonia. Emerg Microbes Infect. 2020; 9:727–732. https://doi.org/10.1080/22221751.2020.1746199 PMID:32196410

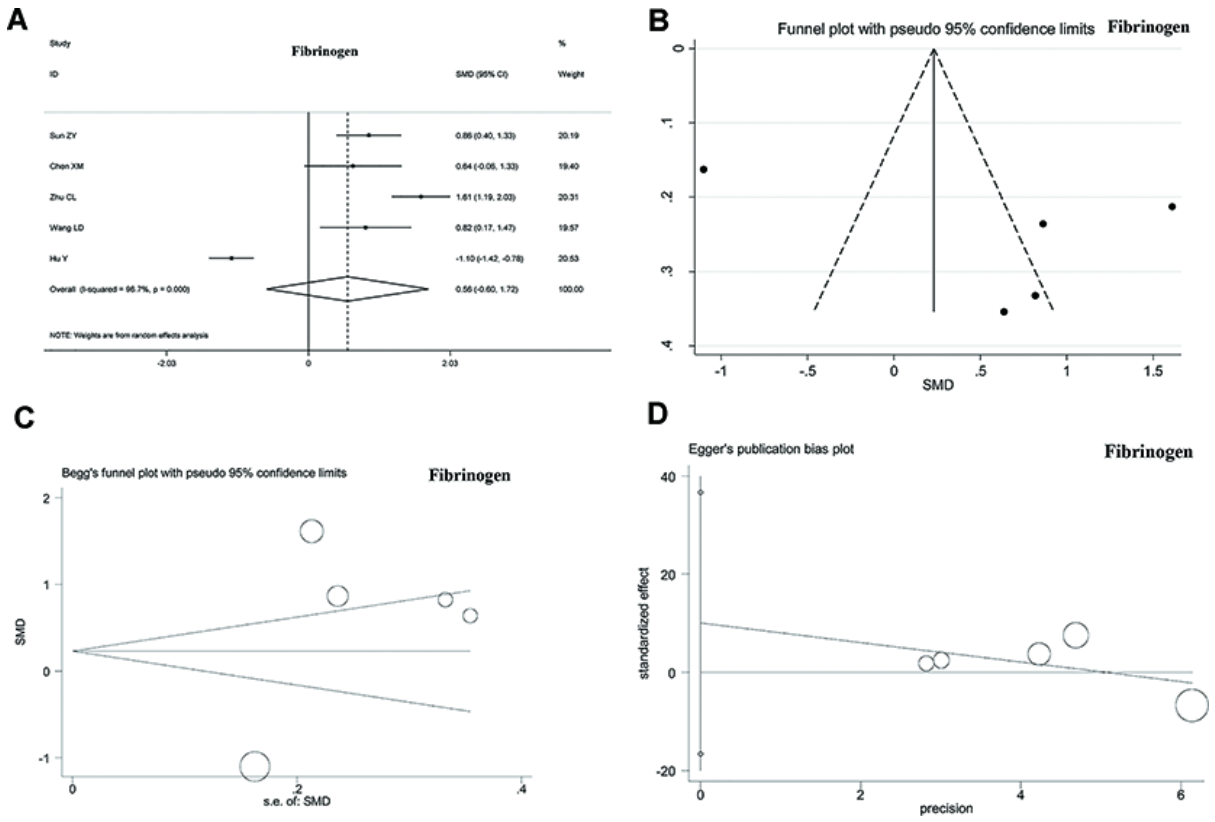
67. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. BMJ. 2009; 339:b2700. https://doi.org/10.1136/bmj.b2700 PMID:19622552

68. Stang A. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. Eur J Epidemiol. 2010; 25:603–05. https://doi.org/10.1007/s10654-010-9491-z PMID:20652370

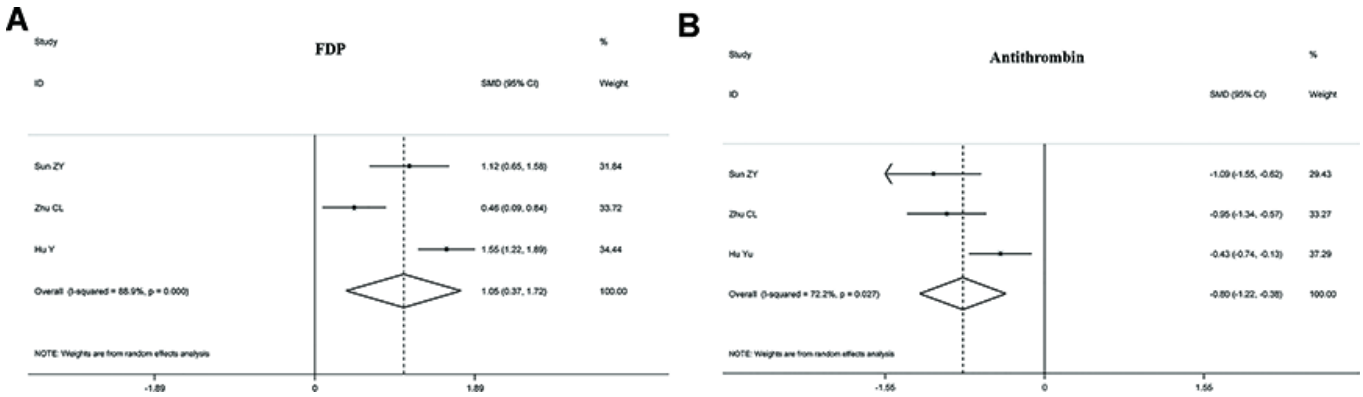
69. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003; 327:557–60. https://doi.org/10.1136/bmj.327.7414.557 PMID:12958120

SUPPLEMENTARY MATERIALS

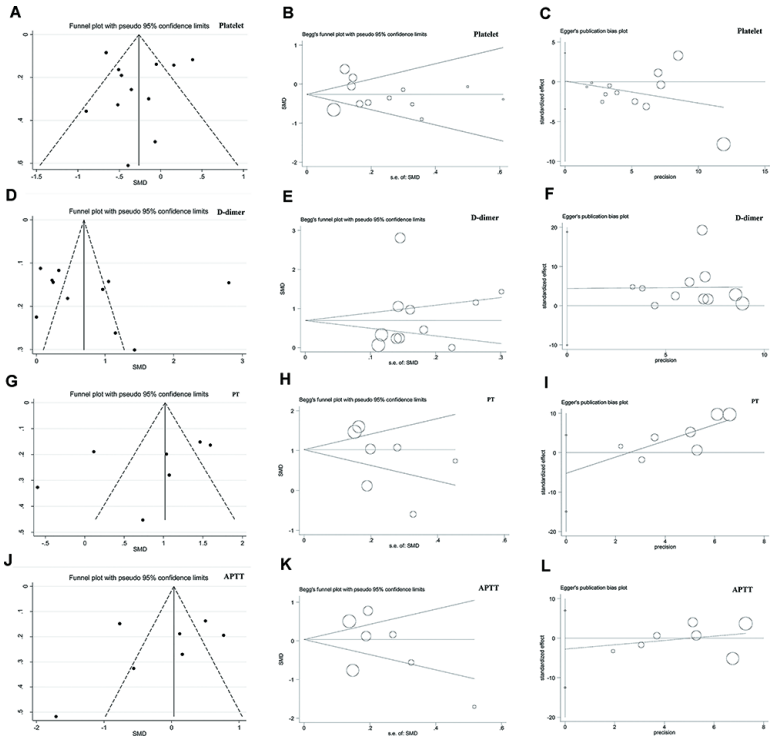
Supplementary Figures



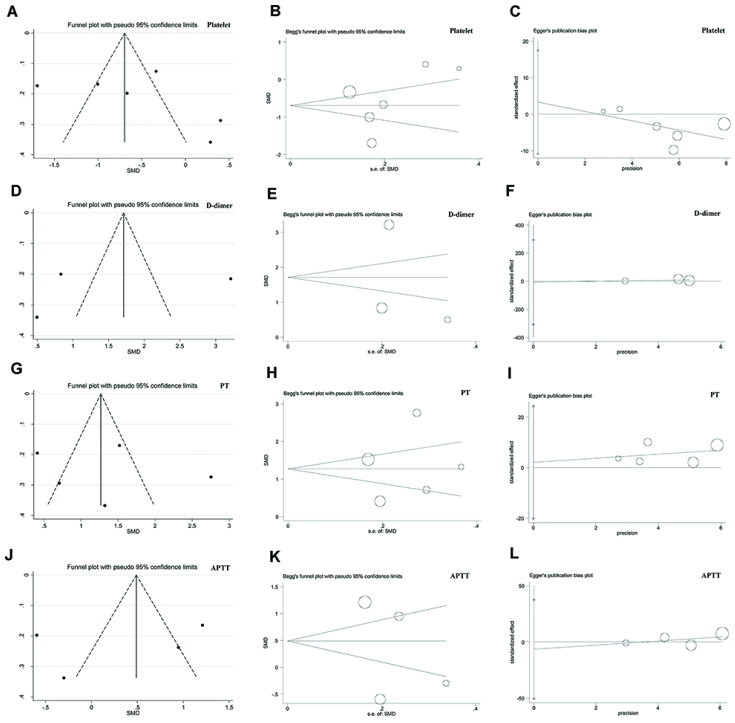
Supplementary Figure 1. Forest plots and publication bias of fibrinogen. Forest plots of pooled standard mean difference and 95% CIs assessing the severity status of COVID-19 patients by fibrinogen. The sizes of the blocks or diamonds represent the weights, and the lengths of the straight lines represent the widths of the 95% CI (**A**) Funnel plot (**B**) Egger's test (**C**) and Begg's (**D**) test assessing the publication bias of fibrinogen.



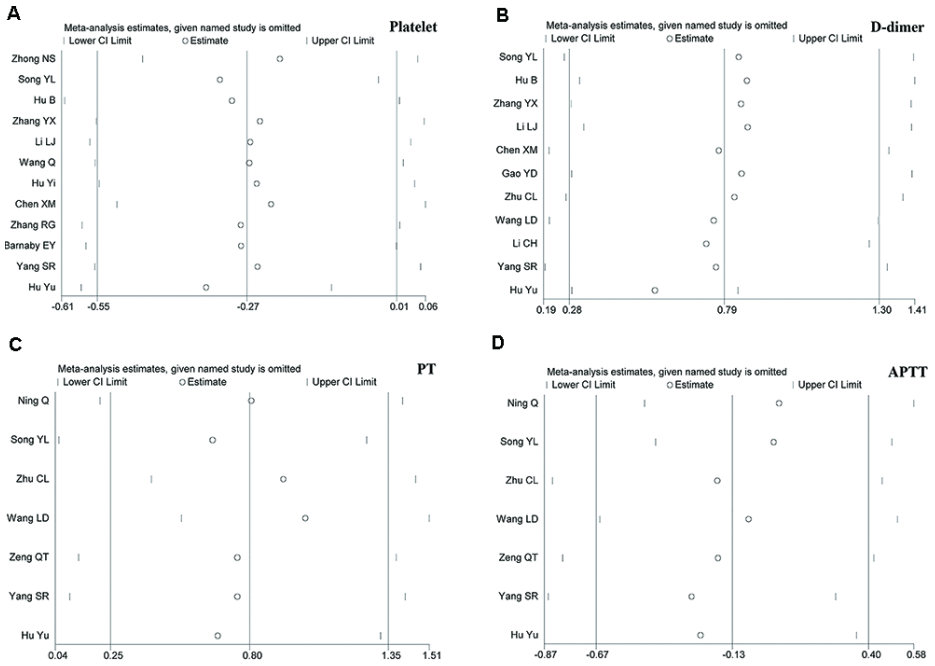
Supplementary Figure 2. Forest plots of pooled standard mean difference and 95% CIs assessing the severity status of COVID-19 patients by fibrin/fibrinogen degradation products (FDP) (**A**) and antithrombin (**B**). The sizes of the blocks or diamonds represent the weights, and the lengths of the straight lines represent the widths of the 95% CIs.



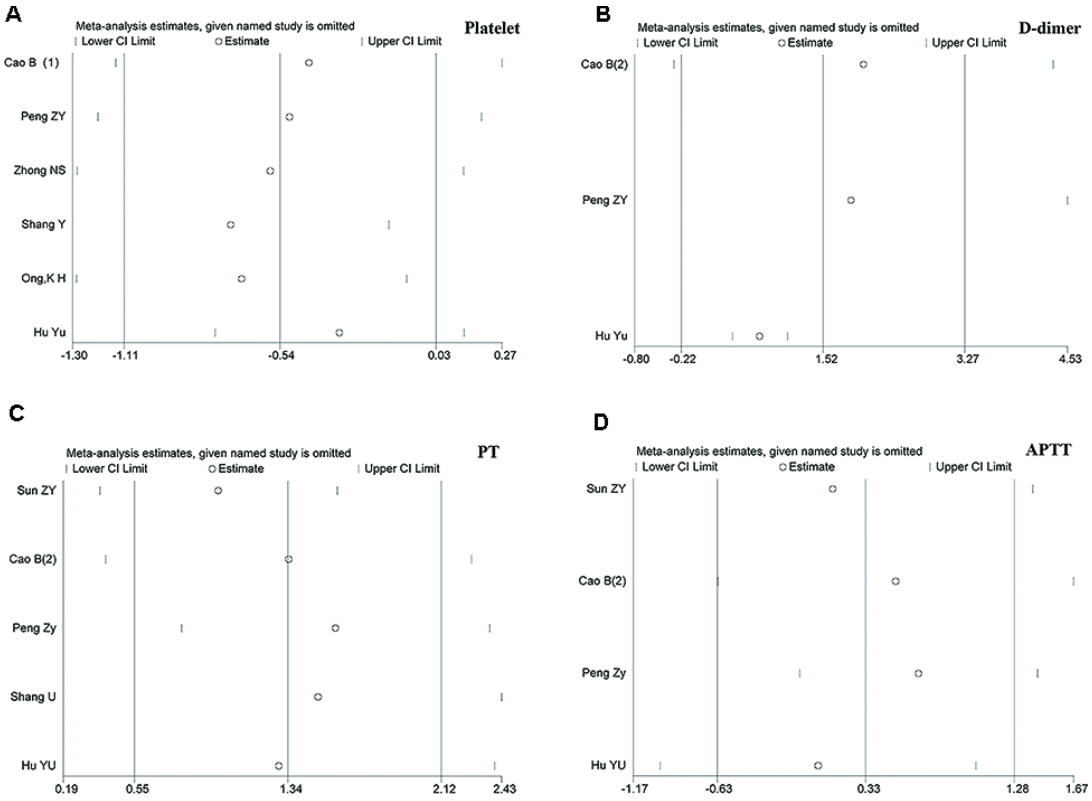
Supplementary Figure 3. Funnel plot, Egger's test and Begg's test assessing the publication bias of platelet (**A**–**C**) D-dimer (**D**–**F**) prothrombin time (PT) (**G**–**I**) and activated partial thromboplastin time (APTT) (**J**–**L**) associated with the severity status, respectively.



Supplementary Figure 4. Funnel plot, Egger's test and Begg's test assessing the publication bias of platelet (**A**–**C**) D-dimer (**D**–**F**) prothrombin time (PT) (**G**–**I**) and activated partial thromboplastin time (APTT) (**J**–**L**) associated with the composite endpoint, respectively.



Supplementary Figure 5. Sensitivity analysis of studies involving platelet (**A**) D-dimer (**B**) prothrombin time (PT) (**C**) and activated partial thromboplastin time (APTT) (**D**) associated with the severity status. None of the articles removed would have a significant effect on the results.



Supplementary Figure 6. Sensitivity analysis of studies involving platelet (**A**) D-dimer (**B**) prothrombin time (PT) (**C**) and activated partial thromboplastin time (APTT) (**D**) associated with the composite endpoint. None of the articles removed would have a significant effect on the results.

Supplementary Tables

Supplementary Table 1. Newcastle-Ottawa quality assessment scale (NOS).

NUM	Study	Selection (****)				Comparability (**)	Exposure (***)			Score
		Case definition adequate	Representativeness of the cases	Selection of controls	Definition of controls		Ascertainment of exposure	Same method of ascertainment for cases and controls	Non-Response rate	
1	Cao B	*	*		*	**	*	*	*	8
2	Sun ZY	*	*		*	**	*	*	*	8
3	Cao B (2)	*	*		*	**	*	*	*	8
4	Ning Q	*	*		*	**	*	*	*	8
5	Peng ZY	*	*		*	**	*	*	*	8
6	Zhong NS	*	*		*	**	*	*	*	8
7	Song YL	*	*		*	**	*	*	*	8
8	Hu B	*	*		*	**	*	*	*	8
9	Zhang YX	*	*		*	**	*	*	*	8
10	Li LJ	*	*		*	**	*	*	*	8
11	Shang Y	*	*		*	**	*	*	*	8
12	Ong, K H	*	*		*	**	*	*	*	8
13	Wang Q	*	*		*	**	*	*	*	8
14	Hu Y	*	*		*	**	*	*	*	8
15	Chen XM	*	*		*	**	*	*	*	8
16	Gao YD	*	*		*	**	*	*	*	8
17	Zhang RG	*	*		*	**	*	*	*	8
18	Zhu CL	*	*	*	*	**	*	*	*	9
19	Wang LD	*	*		*	**	*	*	*	8
20	Zeng QT	*	*		*	**	*	*	*	8
21	Li CH	*	*		*	**	*	*	*	8
22	Barnaby EY	*	*		*	**	*	*	*	8
23	Yang SR	*	*		*	**	*	*	*	8
24	Hu Y	*	*		*	**	*	*	*	8

Supplementary Table 2. Research strategy.

PubMed	**Mesh**: severe acute respiratory syndrome coronavirus 2 COVID-19; spike glycoprotein; COVID-19 virus **Entry Terms**: Wuhan coronavirus; Wuhan seafood market pneumonia virus; COVID19 virus; coronavirus disease 2019 virus; SARS-CoV-2; SARS2; 2019-nCoV; 2019 novel coronavirus; 2019 novel coronavirus infection; COVID19; coronavirus disease 2019; coronavirus disease-19; 2019-nCoV disease; 2019 novel coronavirus disease; 2019-nCoV infection; COVID-19 virus spike glycoprotein; 2019-nCoV spike glycoprotein **Search** ((((((((((((((((((severe acute respiratory syndrome coronavirus 2) OR COVID-19) OR spike glycoprotein, COVID-19 virus) OR Wuhan coronavirus) OR Wuhan seafood market pneumonia virus) OR COVID19 virus) OR coronavirus disease 2019 virus) OR SARS-CoV-2) OR SARS2) OR 2019-nCoV) OR 2019 novel coronavirus) OR 2019 novel coronavirus infection) OR COVID19) OR coronavirus disease 2019) OR coronavirus disease-19) OR 2019-nCoV disease) OR 2019 novel coronavirus disease) OR 2019-nCoV infection) OR COVID-19 virus spike glycoprotein) OR 2019-nCoV spike glycoprotein
Web of Science	**TS=**(severe acute respiratory syndrome coronavirus2 OR COVID-19 OR spike glycoprotein, COVID-19 virus OR Wuhan coronavirus OR Wuhan seafood market pneumonia virus OR COVID19 virus OR coronavirus disease 2019 virus OR SARS-CoV-2 OR 2019-nCoV OR 2019 novel coronavirus OR 2019 novel coronavirus infection OR COVID19 OR coronavirus disease 2019 OR coronavirus disease-19 OR 2019-nCoV disease OR 2019 novel coronavirus disease OR 2019-nCoV infection OR COVID-19 virus spike glycoprotein OR 2019-nCoV spike glycoprotein)
Cochrane Library Scopus	We put “COVID-19” into the Mesh box, but no Mesh terms and Tree were available **Search** ("severe acute respiratory syndrome coronavirus2") OR (COVID-19) OR ("spike glycoprotein, COVID-19 virus") OR ("Wuhan coronavirus")OR("Wuhan seafood market pneumonia virus ")OR(" COVID19 virus") OR ("coronavirus disease 2019 virus ")OR ("SARS-CoV-2") OR ("2019-nCoV ")OR ("2019 novel coronavirus") OR ("2019 novel coronavirus infection") OR (COVID19) OR ("coronavirus disease 2019") OR ("coronavirus disease-19") OR ("2019-nCoV disease ")OR ("2019 novel coronavirus disease") OR ("2019-nCoV infection") OR ("COVID-19 virus spike glycoprotein") OR ("2019-nCoV spike glycoprotein")

Research Paper

Clinical characteristics of chronic liver disease with coronavirus disease 2019 (COVID-19): a cohort study in Wuhan, China

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ABSTRACT

Background: Previous work has described acute liver injury (ALI) in coronavirus disease 2019 (COVID-19) pneumonia patients, However, there is limited analyses available investigating chronic liver disease (CLD) in COVID-19 patients. This study aimed to investigate clinical characteristics and outcomes of CLD confirmed in COVID-19 patients.

Results: A total of 104 cases (each group containing 52 patients) were analyzed in this study. The CLD group showed an average of 14 (10.0~21.2) length of stay (LOS) days, compared to the group without CLD that only showed an average of 12.5 (10~16) LOS days (Relative Risk [RR] = 1.34, 95% CI (1.22~1.48), P<0.001; Adjusted Relative Risk was 1.24 (95% CI: 1.12~1.39)). The CLD group contained a higher mortality rate and slight liver injury. Furthermore, COX regression model analyses suggested that the neutrophil-to-lymphocyte ratio (NLR) was an independent predictor of mortality risk (P < 0.001) in the CLD group. Additionally, a high NLR significantly correlated with a shorter overall survival (P <0.001).

Conclusions: COVID-19 patients also diagnosed with CLD suffered longer LOS, slight liver injuries and a higher mortality when compared to COVID-19 patients without CLD. The NLR was an independent risk factor for in-hospital deaths. Increased expression of NLR was an indicator of poor prognosis in COVID-19 patients with CLD. Thus, COVID-19 patients diagnosed with CLD and who show a higher NLR need additional care.

Methods: A retrospective cohort study was performed at the Wuhan Jin Yin-tan Hospital from February 2, 2020 to April 2, 2020. COVID-19 patients diagnosed with CLD or not diagnosed with CLD were enrolled in this study. The clinical characteristics and outcomes of these patients were compared.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) pneumonia, emerged in Wuhan (Hubei, China), has rapidly spread worldwide, infecting over 3.48 million patients. Previous work has mainly described acute liver injury (ALI) in general COVID-19 pneumonia patients [1–4]. There is little research available that focuses on patients with liver disease, especially chronic liver disease (CLD). Although some studies and reviews had reported that CLD is not associated with severity or mortality of COVID-19, all these used small sample sizes and most likely failed to analyze the characteristics and mortality rates of these patients. To our knowledge, the study presented here is the first to investigate clinical features and outcomes of CLD patients who have been diagnosed with COVID-19 pneumonia in a largest cohort study.

RESULTS

A total 104 patients were analyzed in this study, with both groups containing 52 patients (Table 1). In addition to CLD, a total of 39 (37.5%) patients showed other comorbidities. The median age of the patients analyzed was 59 (SD 12.9) years of age and a total of 39 (37.5%) patients were female. The most common symptoms experienced by the patients included cough (85[81.7%]), expectoration (38[36.5%]), dyspnea (19[18.3%]), and fatigue or myalgia (13 [12.5%]). All patients showed bilateral infiltrates on chest CT, while 92 (88.5%) patients had bilateral infiltrates. A total of 34.6%,35.6% and 5.77% of patients showed elevated ALT, AST and TBil levels, respectively. There was a total of 9 death patients in the CLD group, including 6 patients died of respiratory and circulatory failure, 3 patients died of multiple organ dysfunction syndrome (MODS). There were no significant differences observed in demographics, initial common symptoms, laboratory findings without lymphocyte count, PLT, INR, Glu IL-6 or PCT levels, liver function and treatment when comparing the two groups ($P > 0.05$; Table 1). The CLD group had showed a LOS of 14 (10.0~21.2) days compared to12.5 (10~16) for the non- CLD group (Relative Risk [RR] = 1.34, 95% CI (1.22~1.48), $P<0.001$; Adjusted RR was 1.24(95% CI: 1.12–1.39)) (Table 2). However, no differences in severity outcome were observed between the two groups ((Hazard Ratio [HR] = 1.78, 95% CI (0.80~4.04), P=0.16; Adjusted HR was 1.19, 95% CI(0 .45~3.19), P=0.73) (Table 2). There was no difference in severity ratio between the two groups (39 [37.5%] vs 16 [30.8%], p=0.22). The CLD group showed a higher mortality rate (9 [8.7%] vs 0[0.0%]) and slight liver injuries compared to the non-CLD group. Furthermore, univariate and multivariate COX regression analyses were performed to explore risk factors for death in the CLD group. Univariate survival analyses revealed that age, NLR, GLU and PCT were risk factors for death. However, only the NLR (OR, 1.04; 95% CI, 1.01–1.06) was found to be an independent predictor of death based on the multivariate analysis. (Table 3). To further assess prognostic significance of NLR in CLD patients with COVID-19, Kaplan-Meier survival analysis was performed to analyze overall survival (OS) (cutoff = 4.00). CLD patients with high NLR showed a significantly shorter OS (Figure 1, $P <0.001$). Thus, CLD was not associated with severity, but was associated with LOS, liver injury and mortality in patients diagnosed with COVID-19. Furthermore, NLR was shown to be an independent risk factor and prognostic factor for mortality in CLD patients confirmed to have COVID-19.

DISCUSSION

COVID-19 patients diagnosed with CLD showed a prolong LOS, slightly liver injuries and higher mortality rates compared to general COVID-19 patients. Furthermore, the NLR was found to be an independent risk factor for mortality in COVID-19 patients with CLD. Moreover, increased expression of NLR is an independent indicator of poor prognosis in COVID-19 patients diagnosed with CLD.

Previous work conducted by our team and others has shown that COVID-19 patients were more prone to liver injury whether general patients or critically ill patients [1, 5–11]. In the other hand, a severe outcome of COVID-19 disease was associated with liver dysfunction [12]. Although there are some reports that liver injury is uncommon in these cases [13], CLD should also be analyzed related to COVID-19 risk due to poor immune function. However, for CLD patients, some studies [5] showed that no patient should be receiving ICU care. Similarly, there were no significant differences for AST and ALT between the ICU and non-ICU groups, where both groups showed a normal distribution [5]. There were also no differences in liver function between survivors and non-survivors [14]. At the same time, other studies revealed that COVID-19 patients diagnosed with CLD did not show associations with severity or mortality [6]. This research only included rare CLD patients, where in some studies only a single CLD patient was included. Thus, these studies failed to explore the risk of severity or mortality associated with CLD. Here, we focused on all CLD confirmed COVID-19 cases presented in the Jin Yin-tan Hospital. CLD patients showed a prolonged LOS, slight liver injures and an increased risk for death, but not severity.

Table 1. Demographics and clinical features of patients with COVID-19.

	All patients (N= 104)	Non-CLD (n = 52)	CLD (n = 52)	*P*
Demographics				
Age	59 ± 12.9	59.7 ± 14	58.2 ± 11.7	0.58
sex, n (%)				
Female	39 (37.5)	19 (36.5)	20 (38.5)	1
Male	65 (62.5)	33 (63.5)	32 (61.5)	
Comorbidities, n (%)				
No	65 (62.5)	33 (63.5)	32 (61.5)	1
Yes	39 (37.5)	19 (36.5)	20 (38.5)	
Smoking, n (%)				
No	40 (38.5)	20 (38.5)	20 (38.5)	1
Yes	64 (61.5)	32 (61.5)	32 (61.5)	
Initial common symptoms				
Dyspnoea, n (%)				0.13
No	85 (81.7)	46 (88.5)	39 (75)	
Yes	19 (18.3)	6 (11.5)	13 (25)	
Cough, n (%)				
No	19 (18.3)	9 (17.3)	10 (19.2)	1
Yes	85 (81.7)	43 (82.7)	42 (80.8)	
Expectoration, n (%)				
No	66 (63.5)	28 (53.8)	38 (73.1)	0.07
Yes	38 (36.5)	24 (46.2)	14 (26.9)	
Myalgia or fatigue, n (%)				
No	91 (87.5)	46 (88.5)	45 (86.5)	1
Yes	13 (12.5)	6 (11.5)	7 (13.5)	
Sore throat, n (%)				
No	104 (100)	52 (100)	52 (100)	1
Thoracodynia, n (%)				
No	104 (100)	52 (100)	52 (100)	1
Systolic Pressure	125(117.8,133.3)	125 (118.0, 130.5)	125.5(116.3, 135.5)	0.39
Respiratory Rate	22 (20, 24)	22 (20, 23)	22 (20, 25)	0.51
Laboratory findings				
White blood cell count ($\times 10^9$ cells per L)	5.5 (4.4, 7.36)	5.5 (4.36, 6.86)	5.48 (4.58, 8.02)	0.22
Neutrophil count ($\times 10^9$ cells per L)	3.85(2.96, 5.39)	3.79 (2.96, 5.06)	3.92(2.91, 5.85)	0.69
Lymphocyte count ($\times 10^9$ cells per L)	0.88(0.69, 1.18)	1.02 (0.85, 1.24)	0.79(0.55, 1.04)	< 0.001
MNM ($\times 10^9$ cells per L)	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.39
Hb (g/L)	124(114.0,134.3)	124(114.3, 132.0)	124(114.0,136.3)	0.49
PLT ($\times 10^9$ per L)	211.5(164, 268)	233.5(183.75, 296)	186(155, 230)	<0.001
INR	0.96(0.9, 1.04)	0.94 (0.9, 0.99)	1 (0.92, 1.12)	0.03
Potassium (mmol/L)	4.1 ± 0.6	4 ± 0.6	4.2 ± 0.6	0.18
Sodium (mmol/L)	140.5 (139, 142)	141 (139, 143)	140 (138, 142)	0.13
Cl (mmol/L)	106 (104, 108)	107 (105, 108)	106 (103, 108)	0.08
BUN	4 (3.4, 5.23)	4 (3.3, 5.05)	4.1 (3.5, 5.7)	0.28
Cr (μmol/L)	70 (59.45, 79.98)	70.65(59.45, 82.05)	69.85(59.52, 78.15)	0.69
Glu (mmol/L)	5.8 (5.07, 7.05)	5.65 (5, 6.2)	6.3 (5.27, 7.7)	0.02
CK (U/L)	65(42.75, 185.25)	63 (41.00, 143.25)	73 (47.50, 208.25)	0.23
IL-6 (mmol/L)	8.52(6.52,11.52)	7.77 (6.5, 10.52)	9.58 (7.15, 15.45)	0.04
Infection, n (%)				
No	29 (27.9)	15 (28.8)	14 (26.9)	1
Yes	75 (72.1)	37 (71.2)	38 (73.1)	

PCT, Median (IQR)	0 (0, 0.07)	0 (0, 0.05)	0.05 (0, 0.23)	< 0.001
Chest CT				
Lobi Pulmonis, n (%)				
Unilateral	12 (11.5)	5 (9.6)	7 (13.5)	0.76
Bilateral	92 (88.5)	47 (90.4)	45 (86.5)	
Ground-glass opacity, n (%)				
No	45 (43.3)	23 (44.2)	22 (42.3)	1
Yes	59 (56.7)	29 (55.8)	30 (57.7)	
ALT (IU/L, baseline)	36.5(22.75,63.25)	42.5 (22.75, 68)	36 (24.25, 57.5)	0.62
AST (IU/L, baseline)	33 (25, 50)	32 (25, 46.5)	36.5 (25.75, 51.5)	0.4
Total bilirubin(μmol/L)	12.85 (10.38, 16.22)	11.95 (10.2, 14.12)	13.3 (10.95, 17.5)	0.1
ALB(g/L)	32 ± 4.2	31.3 ± 3.5	32.7 ± 4.7	0.08
PT(s)	11.25 (10.5, 12)	11 (10.5, 11.7)	11.65 (10.57, 12)	0.05
PTA	108.35 (89, 127.55)	111 (92.78, 130.6)	105.55 (88.4,124.35)	0.52
Treatment				
Antibiotic therapy, n (%)				
No	18 (17.3)	7 (13.5)	11 (21.2)	0.44
Yes	86 (82.7)	45 (86.5)	41 (78.8)	
Use of corticosteroid, n (%)				
No	79 (76)	43 (82.7)	36 (69.2)	0.17
Yes	25 (24)	9 (17.3)	16 (30.8)	
Oxygen support, n (%)				
No	19 (18.3)	7 (13.5)	12 (23.1)	0.31
Yes	85 (81.7)	45 (86.5)	40 (76.9)	
Ventilation, n (%)				
Non-invasive ventilation	7 (6.7)	2 (3.8)	5 (9.6)	
Invasive mechanical ventilation	6 (5.8)	1 (1.9)	5 (9.6)	0.1
NO ventilation	91 (87.5)	49 (94.2)	42 (80.8)	
Prone position ventilation, n (%)				
No	103 (99)	52 (100)	51 (98.1)	1
Yes	1 (1)	0 (0)	1 (1.9)	
ECMO, n (%)				
No	104 (100)	52 (100)	52 (100)	1
Nebulization inhalation, n (%)				
No	99 (95.2)	50 (96.2)	49 (94.2)	1
Yes	5 (4.8)	2 (3.8)	3 (5.8)	
Vasoconstrictor, n (%)				
No	103 (99)	52 (100)	51 (98.1)	1
Yes	1 (1)	0 (0)	1 (1.9)	
Immunoglobulin therapy, n (%)				
No	86 (82.7)	47 (90.4)	39 (75)	0.07
Yes	18 (17.3)	5 (9.6)	13 (25)	

Abbreviations: CLD, Chronic liver disease; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate; INR, International Normalized Ratio; PT, Prothrombin time; *PLT*, *blood platelet; CK, creatine kinase;* BUN, blood urea nitrogen; *RR*: Respiratory Rate; MNM: monocyte counts; IL-6, interleukin-6; ECMO, extracorporeal membrane oxygenation.

To the best of our knowledge, this is the first study focusing on COVID-19 patients diagnosed with CLD. The COVID-19 with CLD group showed an increased lymphocyte count as well as increased IL-6 and PCT levels, suggesting pathogenic effects from excessive inflammation in acute lung injury caused by COVID-19 infection. Inflammation may reflect disease severity and defects in innate immune regulation, especially in CLD patients who have poor immune function [13]. At the same time, blood sugar levels were elevated to support

Table 2. Unadjusted and adjusted risk ratios of LOS, severity, and mortality in COVID-19.

Outcome	β	Unadjusted risk ratio	95% CI	*P*	β	Adjusted risk ratio	95% CI	*P*
LOS Outcome								
	0.30	1.34	(1.22~1.48)	<0.01	0.22	1.24	1.12~1.39	<0.001
Severity Outcome								
	0.58	1.78	0.80~4.04	0.16	0.17	1.19	0.45~3.19	0.73
Mortality Rate Outcome								
			CLD (n = 52)			Non-CLD (n = 52)		*P*
	Survivors		43 (82.7)			52 (100)		<0.01
	Death		9 (17.3)			0 (0)		
Liver Injury Outcome								
		Total (N = 104)	CLD (n = 52)			Non-CLD (n = 52)		*P*
ALT		37 (24.75, 57.25)	39.5 (28.75, 55.5)			33.5 (23, 62)		0.47
AST		28.5 (19, 38.75)	30 (23, 49.5)			24 (17.75, 34.25)		< 0.001
TBil		10.05 (6.7, 15.12)	13.9 (7.18, 20.8)			8.6 (6.7, 11.93)		< 0.001

(CLD group verse Non-CLD group).

Table 3. Unadjusted and adjusted risk factors of mortality for CLD group.

	β	Unadjusted hazard ratio	95%CI	*P*	β	Adjusted hazard ratio	95%CI	*P*
Age	2.13493	8.46	1.73~41.37	0.0084				
NLR	0.046	1.05	1.02~1.07	<0.001	0.03624	1.04	1.01~1.06	<0.001
GLU	0.24	1.27	1.03~1.57	0.028				
IL-6	0.005009	1.01	0.97 ~1.05	0.799				
PCT	2.28	9.74	1.85~51.20	<0.001				
INR	0.04832	0.95	0.45~2.02	0.9				

NLR, neutrophil-to-lymphocyte ratio.

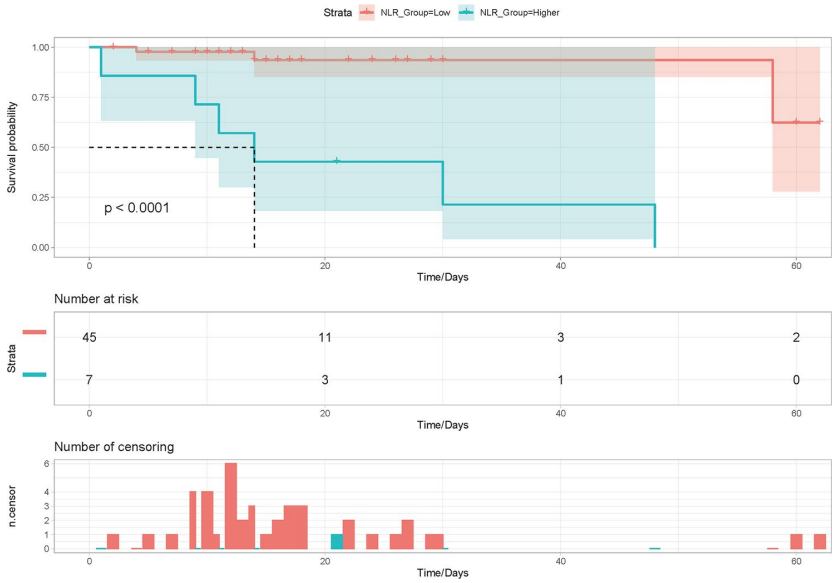

Figure 1. Kaplan-Meier analysis of overall survival (OS) according to NLR expression in CLD with COVID-19 infection patients.

the specific energetic demands needed by inflammatory responses [15, 16]. This work also revealed that the CLD group had relatively low PLT and increased total bilirubin and INR levels, supporting hepatic dysfunction in the activation of coagulative and fibrinolytic, which consistent with previous studies [13, 17] In this study, risk of CLD was found to be related to LOS in COVID-19. Compared to patients without CLD, the patient group diagnosed with COVID-19 and CLD showed a prolonged LOS. With a prolonged LOS, CLD may improve the risk of nosocomial infections (NIs), which may result in a worse prognosis. Similarly, the CLD group showed an increased mortality rate and higher incidence of liver injury. Interestingly, although the risk of CLD was positively associated with LOS, liver function risk and mortality, it was negatively associated to severity which was consistent with other studies [6, 13]. We performed additional work to explore risk factors related to mortality in the CLD group. The most important finding was that the NLR was associated with mortality and severity, suggesting it as a potential indicator for poor prognosis in CLD patients diagnosed with COVID-19 infection. The high NLR accounts for increased neutrophil and decreased lymphocyte counts, reflecting systemic inflammation. Systemic inflammation is believed to play an important role in the severity of virus-induced disease, such as COVID-19. Meanwhile, patients diagnosed with CLD have poor immune function. Thus, CLD may aggravate the dysregulation of the immune response associated with COVID-19 [18]. An easily accessible and less costly biological marker for systemic inflammatory diseases, NLR has been reported as an independent risk factor for the severity and mortality in COVID-19 [3, 18, 19], especially in elder or male patients [19]. Since NLR could be quickly calculated based on a blood routine test on admission, clinicians may identify high risk COVID-19 patients at an early stage. Thus, treatments can be modified accordingly to reduce the in-hospital death. In particular, this study confirmed the risk of NLR associated with mortality in CLD patients.

This study also exhibits some limitations. (a) The sample size was limited to a single-center hospital, clinical characteristics COVID-19 patients, diagnosed with CLD, should be verified with a randomized controlled trial enrolling more patients. (b) The diagnosis of CLD needs systematic testing such as a biopsy of the liver, blood tests and imaging including ultrasound. This study was unable to widely diagnose all CLD cases through systematic testing when the COVID-19 outbreak occurred in Wu Han since there were limited medical resources. However, CLD was diagnosed based on written medical and *oral health records with the goal to minimize bias* of diagnosis. (c) The study population included older COVID-19 patients diagnosed with CLD, suggesting that these conclusions may not be applicable to younger patients. In the future, a larger multicenter study analyzed CLD patients diagnosed with COVID-19 is needed to further understand the pathological mechanisms behind CLD associated with COVID-19. This would contribute to further knowledge defining the clinical characteristics and outcome for these patients.

CONCLUSIONS

This retrospective study revealed that COVID-19 patients diagnosed with CLD showed a longer LOS, slight liver injuries and higher mortality compared to general COVID-19 patients. The NLR was found to be an independent risk factor for in-hospital deaths. Increased expression of NLR was found to be a potential indicator for poor prognosis in COVID-19 patients diagnosed with CLD. Thus, CLD patients with COVID-19 who have a higher NLR should be critically cared for.

MATERIALS AND METHODS

Study design, participants and data collection

The retrospective cohort study presented here included all CLD and random non-CLD patients at Wuhan Jin Yin-tan Hospital. Wuhan Jin Yin-tan Hospital (Wuhan Isolation Hospital) is known to have treated the largest number of COVID-19 patients. All enrolled patients were treated from February 2, 2020 to April 2, 2020. The diagnostic and treatment criteria of COVID-19 and its severity were based on guidelines provided by the WHO and China Trial Seventh Edition. Patients diagnosed with acute liver injury or who showed incomplete medical records were excluded from this study. Clinical data such as demographics, initial symptoms, laboratory findings, chest CT pneumonia compromise and treatment was reviewed using digital medical records by the Fujian Medical Team to aid Wuhan Jin Yin-tan Hospital. Patients were divided into two groups including the COVID-19 with CLD group and non-CLD group. CLD was defined as a progressive deterioration of liver functions, leading to fibrosis and cirrhosis of liver parenchyma. It refers to liver disease at least 6 months. CLD consists of diverse liver pathologies including hepatocellular carcinoma, liver cirrhosis, and inflammation (chronic hepatitis). Our team diagnosed CLD based on clinical features. The COVID-19 with CLD group included all CLD patients that were diagnosed with chronic viral hepatitis B and C, autoimmune liver disease, cryptogenic liver cirrhosis, NAFLD, methotrexate related liver fibrosis and alcoholic liver disease. At the same time, we used computer-generated random same size to enroll

non-CLD group during the same period. We analyzed the clinical characteristics of all patients, then compared the baseline information and the outcome of LOS, severity, mortality rate and liver function. This retrospective cohort study approved by Ethics Commission of Jin Yin-tan hospital, Wuhan (KY-2020–55.01).

Statistical analysis

The mean ± SD or median (IQR) value and number (%) were used to descriptive data of continuous and categorical variables. For continuous variables, independent group t tests were used to compare the two group or Mann–Whitney test was performed when data were normally distributed. For categorical variables, the χ^2 or Fisher exact tests were performed to compared the two groups. Furthermore, Poisson regression was used to verify independent risk of CLD for LOS. Stepwise Logistic Regression models were used to test independent risks of CLD for severity. Cox models were used to calculate the hazard ratio of mortality in the CLD group. Kaplan-Meier survival analysis was used to analyze overall survival (OS) of the CLD group based on neutrophil-to-lymphocyte ratio (NLR) levels. R project (version 3.6.0) was used to perform all statistical analyses. Statistical significance was recognized at a P value of 0.05 or less.

AUTHOR CONTRIBUTIONS

ZR Yang designed study, analyzed and revised the manuscript; Chaowei Li and Qingshi Chen collected data and wrote the manuscript; Jianwen Wang, Huasong Lin, Yalan Lin, Jinhuang Lin, Fangzhan Peng and Jiangmu Chen searched the literature.

CONFLICTS OF INTEREST

The authors disclose no conflicts of interest.

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REFERENCES

1. Xie H, Zhao J, Lian N, Lin S, Xie Q, Zhuo H. Clinical characteristics of non-ICU hospitalized patients with coronavirus disease 2019 and liver injury: a retrospective study. Liver Int. 2020; 40:1321–26. https://doi.org/10.1111/liv.14449 PMID:32239591

2. Xu L, Liu J, Lu M, Yang D, Zheng X. Liver injury during highly pathogenic human coronavirus infections. Liver Int. 2020; 40:998–1004. https://doi.org/10.1111/liv.14435 PMID:32170806

3. Zhang Y, Zheng L, Liu L, Zhao M, Xiao J, Zhao Q. Liver impairment in COVID-19 patients: a retrospective analysis of 115 cases from a single centre in Wuhan city, China. Liver Int. 2020. [Epub ahead of print]. https://doi.org/10.1111/liv.14455 PMID:32239796

4. Zhao D, Yao F, Wang L, Zheng L, Gao Y, Ye J, Guo F, Zhao H, Gao R. A comparative study on the clinical features of COVID-19 pneumonia to other pneumonias. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa247 PMID:32161968

5. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

6. Lippi G, de Oliveira MH, Henry BM. Chronic liver disease is not associated with severity or mortality in coronavirus disease 2019 (COVID-19): a pooled analysis. Eur J Gastroenterol Hepatol. 2020. [Epub ahead of print]. https://doi.org/10.1097/MEG.0000000000001742 PMID:32282549

7. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, Xie C, Ma K, Shang K, Wang W, Tian DS. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa248 PMID:32161940

8. Ruan Q, Yang K, Wang W, Jiang L, Song J. Correction to: clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Med. 2020; 46:1294–97. https://doi.org/10.1007/s00134-020-06028-z PMID:32253449

9. Ji D, Zhang D, Xu J, Chen Z, Yang T, Zhao P, Chen G, Cheng G, Wang Y, Bi J, Tan L, Lau G, Qin E. Prediction for progression risk in patients with COVID-19 pneumonia: the CALL score. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa414 PMID:32271369

10. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course

and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81.
https://doi.org/10.1016/S2213-2600(20)30079-5
PMID:32105632

11. Chen R, Liang W, Jiang M, Guan W, Zhan C, Wang T, Tang C, Sang L, Liu J, Ni Z, Hu Y, Liu L, Shan H, et al, and Medical Treatment Expert Group for COVID-19. Risk factors of fatal outcome in hospitalized subjects with coronavirus disease 2019 from a nationwide analysis in China. Chest. 2020; 158:97-105.
https://doi.org/10.1016/j.chest.2020.04.010
PMID:32304772

12. Youssef M, Hussein M, Attia AS, Elshazli R, Omar M, Zora G, Farhoud A, Elnahla A, Shihabi A, Toraih E, Fawzy M, Kandil E. COVID-19 and liver dysfunction: a systematic review and meta-analysis of retrospective studies. J Med Virol. 2020; 10:1002.
https://doi.org/10.1002/jmv.26055
PMID:32445489

13. Bangash MN, Patel J, Parekh D. COVID-19 and the liver: little cause for concern. Lancet Gastroenterol Hepatol. 2020; 5:529–30.
https://doi.org/10.1016/S2468-1253(20)30084-4
PMID:32203680

14. Huang J, Cheng A, Kumar R, Fang Y, Chen G, Zhu Y, Lin S. Hypoalbuminemia predicts the outcome of COVID-19 independent of age and co-morbidity. J Med Virol. 2020; 10:1002.
https://doi.org/10.1002/jmv.26003
PMID:32406952

15. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, Matos C, Bruss C, Klobuch S, Peter K, Kastenberger M, Bogdan C, Schleicher U, et al. LDHA-associated lactic acid production blunts tumor immunosurveillance by T and NK cells. Cell Metab. 2016; 24:657–71.
https://doi.org/10.1016/j.cmet.2016.08.011
PMID:27641098

16. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. Science. 2013; 342:1242454.
https://doi.org/10.1126/science.1242454
PMID:24115444

17. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69.
https://doi.org/10.1001/jama.2020.1585
PMID:32031570

18. Liu Y, Du X, Chen J, Jin Y, Peng L, Wang HH, Luo M, Chen L, Zhao Y. Neutrophil-to-lymphocyte ratio as an independent risk factor for mortality in hospitalized patients with COVID-19. J Infect. 2020; 81:e6–12.
https://doi.org/10.1016/j.jinf.2020.04.002
PMID:32283162

19. Liu J, Liu Y, Xiang P, Pu L, Xiong H, Li C, Zhang M, Tan J, Xu Y, Song R, Song M, Wang L, Zhang W, et al. Neutrophil-to-lymphocyte ratio predicts critical illness patients with 2019 coronavirus disease in the early stage. J Transl Med. 2020; 18:206.
https://doi.org/10.1186/s12967-020-02374-0
PMID:32434518

Research Paper

Clinical course and characteristics of patients with coronavirus disease 2019 in Wuhan, China: a single-centered, retrospective, observational study

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ABSTRACT

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for the coronavirus disease 2019(COVID-19) pandemic. Despite the extensive studies aiming to understand the pathology of COVID-19, the clinicopathological characteristics and risk factors associated with COVID-19 remain mostly unclear. In this study, we assessed the clinical course and features of COVID-19 patients.

Findings: There were 59 patients (54.1%) that had no fever. One-hundred (91.7%) patients required oxygen therapy, which improved percutaneous oxygen saturation (SpO_2). Seventy-two (66.1%) patients aged over 60; these patients were more likely to develop respiratory symptoms. Only 13 (11.9%) patients were positive for anti-SARS-CoV-2 antibodies, SARS-CoV-2 nucleic acid, and computed tomography (CT) findings. We found significant differences in age, respiratory symptoms, and heart rates between patients with and without underlying conditions.

Conclusions: Our findings suggest that oxygen plays an important role in the treatment of COVID-19 patients and that age and underlying diseases are significant risk factors for COVID-19. Most COVID-19 patients have no fever, and CT provides higher detection rates than antibody- and nucleic acid-based detection methods.

Methods: We analyzed data from 109 confirmed COVID-19 cases. We compared the clinicopathological characteristic of patients stratified according to age and underlying diseases, as well as assessed the detection rates of different diagnostic methods.

INTRODUCTION

In December 2019, an outbreak of severe acute respiratory syndrome coronavirus 2(SARS-CoV-2) in Wuhan, China, marked the beginning of the coronavirus disease 2019(COVID-19) pandemic [1–3]. As of April 8, 2020, the number of confirmed cases has risen to 1.35 million worldwide.[4] COVID-19 often present with persistent fever, cough, chest distress, dyspnea, and sore throat. Some patients also develop gastrointestinal symptoms, including diarrhea, while other patients have no obvious symptoms, making the virus spread containment extremely challenging [5, 6]. In COVID-19 patients, lung computed tomography (CT) findings include bilateral scattered patchy ground-glass density shadows and consolidation stripe shadows in both lungs [7, 8].

Despite the extensive efforts of the last months to understand the pathology of COVID-19 and identify

therapeutic targets, the clinicopathological characteristics and risk factors associated with COVID-19 remain largely unclear. In this study, we investigated the clinicopathological characteristics and treatment outcomes in 109 patients diagnosed with COVID-19. The findings reported herein provide a better understanding of the clinical course, treatment efficacy, and risk factors in COVID-19 patients, providing a step forward toward the development of novel strategies to contain the pandemic.

RESULTS

Patient demographics and characteristics

In this study, we included 109 patients diagnosed with COVID-19 from February 13, 2020, to February 29, 2020, at Wuhan Union Hospital. The demographics and characteristics of these patients are summarized in Supplementary Table 1. The median age of all patients was 63 (range, 29-97), and 72 (66.1%) patients were aged over 60. There were 51 (46.8%) female patients and 58 (53.2%) male patients. Fourth-seven (43.1%) patients had chronic diseases. Among all patients, 100 (91.7%) required oxygen therapy, after which percutaneous oxygen saturation (SpO_2) values returned to physiological levels ($SpO_2 \geq 94\%$).

Clinical features

The clinical features of COVID-19 patients are summarized in Supplementary Table 2. Common symptoms included increased heart rate (n = 57; 52.3%), cough (n = 56; 51.4%), mild fever (37.3°C-38°C), and chest tightness (n = 36; 33.0%); high fever (39°C-40°C) was observed in three patients (2.8%). Only 50 (45.9%) patients presented with fever, while the remaining 59 (54.1%) patients did not develop fever throughout the disease course. Among the patients who developed fever, the median body temperature was 37.8°C (ranges, 37.3°C -40°C), and the median fever duration was 2.5 days (ranges, 1-8 days). 95 (87.1%) patients presented with respiratory symptoms at the time of diagnosis, and in 31 (28.5%) patients, respiratory symptoms continued even after O_2 supplement. Of the 47 (43.1%) patients who had chronic diseases, 41 (87.2%) had respiratory symptoms at the time of diagnosis, and 13 (31.7%) had respiratory symptoms after oxygen therapy. A total of 31 (28.4%) patients were diagnosed with abnormal SpO_2; in these patients, SpO_2 values returned to physiological levels after O_2 supplement.

Among all patients with underlying diseases, 17 (36.2%) were O_2 unsaturated on admission. The median age of patients with respiratory symptoms after oxygen therapy was 65.5 (ranges, 29-97), whereas the median age of patients without respiratory symptoms after O_2 supplement was 62 (range, 29-91). The median age of patients with respiratory symptoms requiring oxygen therapy was 73 (range, 60-83), whereas that of patients not requiring oxygen therapy was 65 (range, 58-65) (Supplementary Table 1).

In this study, we also compared the demographics and clinical characteristics of patients with and without chronic diseases (Table 1). While the median age of patients with chronic diseases was 69 (range, 38-97), that of patients without underlying conditions was 60 (range, 29-91) (Table 1); this difference was statistically significant. Compared with patients with chronic diseases, respiratory symptoms were less frequent, and heart rates were lower in patients without underlying conditions ($P<0.0284$ and $P<0.0001$, respectively; Table 1). No significant differences in gender or other clinical characteristics were observed between patients with chronic diseases and those without chronic conditions (Table 1). Significant factors identified by univariate analyses (age, respiratory symptoms, and heart rate) were included in multivariate analyses; age and heart rate were identified as significant risk factors of chronic diseases (Table 1).

Laboratory parameters and imaging findings

The results of laboratory examination and computed tomography (CT) in COVID-19 patients are shown in Figure 1 and Supplementary Table 3. Although 101 (92.6%) of the patients had positive CT findings, and 91 (83.5%) were positive for anti-SARS-CoV-2 antibodies, only 24 (22.0%) were positive for SARS-CoV-2 nucleic acid. Only 13 (11.9%) of the cases were positive for anti-SARS-CoV-2 antibodies, SARS-CoV-2 nucleic acid, and CT findings. Eighty-three (76.1%) cases were positive for both anti-SARS-CoV-2 antibodies and CT findings, and 18 (16.5%) were positive for SARS-CoV-2 nucleic acid and CT findings.

Eighty-two (75.3%) of the patients with anti-SARS-CoV-2 antibodies presented with respiratory symptoms at the time of diagnosis, whereas 9 (8.2%) had no respiratory symptoms at diagnosis (Table 2). Eighty-eight (80.7%) patients with CT findings had respiratory symptoms at the time of diagnosis, while 13 (11.9%) cases with CT findings had no respiratory symptoms at diagnosis. Importantly, the number of patients who had respiratory symptoms after oxygen therapy was lower among individuals with anti-SARS-CoV-2 antibodies and CT findings. Among the patients with anti-SARS-CoV-2 antibodies and who received O_2 supplement, 24 (29.3%) had respiratory symptoms even after oxygen therapy. Additionally, among the patients with CT findings and who received O_2 supplement, 27 (29.0%) had respiratory

Table 1. Unvariate and multivariate analysis of patients with or without chronic diseases.

	All patients	Chronic diseases	Non-chronic diseases	Univariate P value	Multivariate P value
Sex	109(100)	47(43.1)	62(56.9)		
Female	51(46.8)	20(18.3)	31(28.4)	0.4403	
Male	58(53.2)	27(24.6)	31(28.4)		
Age, median(range)	63(29-97)	69(38-97)	60(29-91)	<0.0001	<0.0001
≤39	6(5.5)	1(0.9)	5(4.6)	0.0061	
40-59	31(28.4)	7(6.4)	24(22.0)		
60-79	56(51.4)	28(25.7)	28(25.7)		
≥80	16(14.6)	11(10.0)	5(4.6)		
Initial respiratory symptoms[a]					
Yes	95(87.2)	41(37.6)	54(49.5)	0.9831	
No	14(12.8)	6(5.5)	8(7.3)		
Respiratory symptoms after O_2 supplement					
Yes	31(28.4)	18(16.5)	23(21.1)	0.2676	
No	69(63.3)	23(21.1)	46(42.2)		
Median temperature,℃	37.2(36.6-40)	37.2(36.6-39.8)	37.2(36.8-40)	0.6853	
Initial median SpO2 value (%)	95(64-98)	95(91-97)	102(86-135)	0.4961	
Median SpO2 value after O_2 supplement (%)	98(96-100)	98(96-100)	98(96-100)	0.9876	
Median heart rate, beats per min	101(84-135)	100(84-126)	95(80-98)	<0.0001	<0.0001
Median respiratory rate, breaths per min	22(20-34)	22(20-34)	21(20-28)	0.0284	

Data are n (%), unless otherwise specified. Abbreviations: COVID-19, coronavirus disease 2019; SpO2, Percutaneous oxygen saturation; O_2, oxygen.

[a]including cough, sore throat, short of breath, chest tightness, expectoration and dyspnea.

symptoms after oxygen therapy. However, among the patients with positive SARS-CoV-2 RT-PCR results, respiratory symptoms continued after oxygen therapy in 12 (52.2%) of them.

DISCUSSION

SARS-CoV-2 is the third coronavirus discovered so far; it is considerably more infectious than SARS-CoV and MERS-CoV [9–12], leading to the rapid spread of COVID-19 across almost every country [13, 14]. In this study, we reported on the clinicopathological characteristics and clinical course of 109 COVID-19 patients. The median age was 63 (ranges 29-97), and 72 (66.1%) patients aged over 60. Forty-seven (43.1%) patients had underlying conditions. Among all patients, 100 (91.7%) received oxygen therapy, after which SpO_2 values returned to physiological levels, suggesting that O_2 supplementation played an important role in improving the condition of patients infected with SARS-CoV-2.

Interestingly, more than half of the patients (54.1%) did not develop fever, in contrast to the study by Goyal P et al., which reported that 77.1% of patients had a fever [15]. Interestingly, Zhiliang Hu et al. reported that only 20.8% of COVID-19 patients developed fever [16], highlighting the high variation in the symptoms of COVID-19 across cohorts. We believe that the fact that SARS-CoV-2 has been circulating for more than half a year has contributed to the attenuation of the symptoms caused by the virus. Additionally, as some COVID-19 patients remain asymptomatic, routine testing using antibody detection tests, nucleic acid testing, and chest CT, should be implemented in every country to contain the pandemic. Among patients who had a fever, the median body temperature was 37.8°C, and the median duration of the fever was 2.5 days; therefore, SARS-CoV-2 infection screening solely by measuring body temperature is insufficient. Additionally, a few patients did not have respiratory symptoms on admission but developed such symptoms later during the disease course. This finding highlights the need for early thorough clinical examination, CT scan, and laboratory testing in suspected cases. Moreover, self-isolation for at least 14 days is crucial for the prevention of community spread of SARS-CoV-2.

In our cohort, more than half of COVID-19 patients were elderly (over 60 years old), consistent with findings from previous studies [1, 17]; hence, we conclude that elderly patients are more likely to be infected with SARS-CoV-2 and that strict measures should be implemented to prevent the spear of the virus in the elderly. Our findings also revealed that people over 60 years old were more likely to develop respiratory symptoms, including abnormal SpO_2, compared with younger individuals. Furthermore, the elderly were less likely to improve after oxygen therapy, further supporting the need for close monitoring of COVID-19 patients aged more than 60. We observed significant differences in age, respiratory symptoms, and heart rates between patients with chronic diseases and those without underlying conditions, suggesting that these factors may indicate the presence of chronic diseases.

It has been reported that patients with underlying diseases were more likely to contract SARS-CoV-2 [17, 18]. In this study, we found that 43.1% of the patients had chronic diseases. After oxygen therapy, the respiratory symptoms continued in one-third of the patients with underlying conditions. However, SpO_2 returned to the physiological levels in all patients after oxygen therapy, pinpointing the importance of O_2 supplement for the treatment of COVID-19 patients with underlying conditions.

The combination of laboratory examination and CT scans plays an important role in the diagnosis of COVID-19. In this cohort, although 92.6% of patients received CT-based diagnosis, and 83.5% were positive for anti-SARS-CoV-2 antibodies, only 22.0% of them were positive for SARS-CoV-2 nucleic acid. Importantly, only 11.9% of the patients were positive for anti-SARS-CoV-2 antibodies, SARS-CoV-2 nucleic acid, and CT diagnosis. Previous studies have shown that some COVID-19 patients were negative for anti-SARS-CoV-2 antibodies and viral nucleic acid at early stages [19]. CT provided a higher detection rate than laboratory examination, highlighting the importance of CT imaging to confirm SARS-CoV-2 infection.

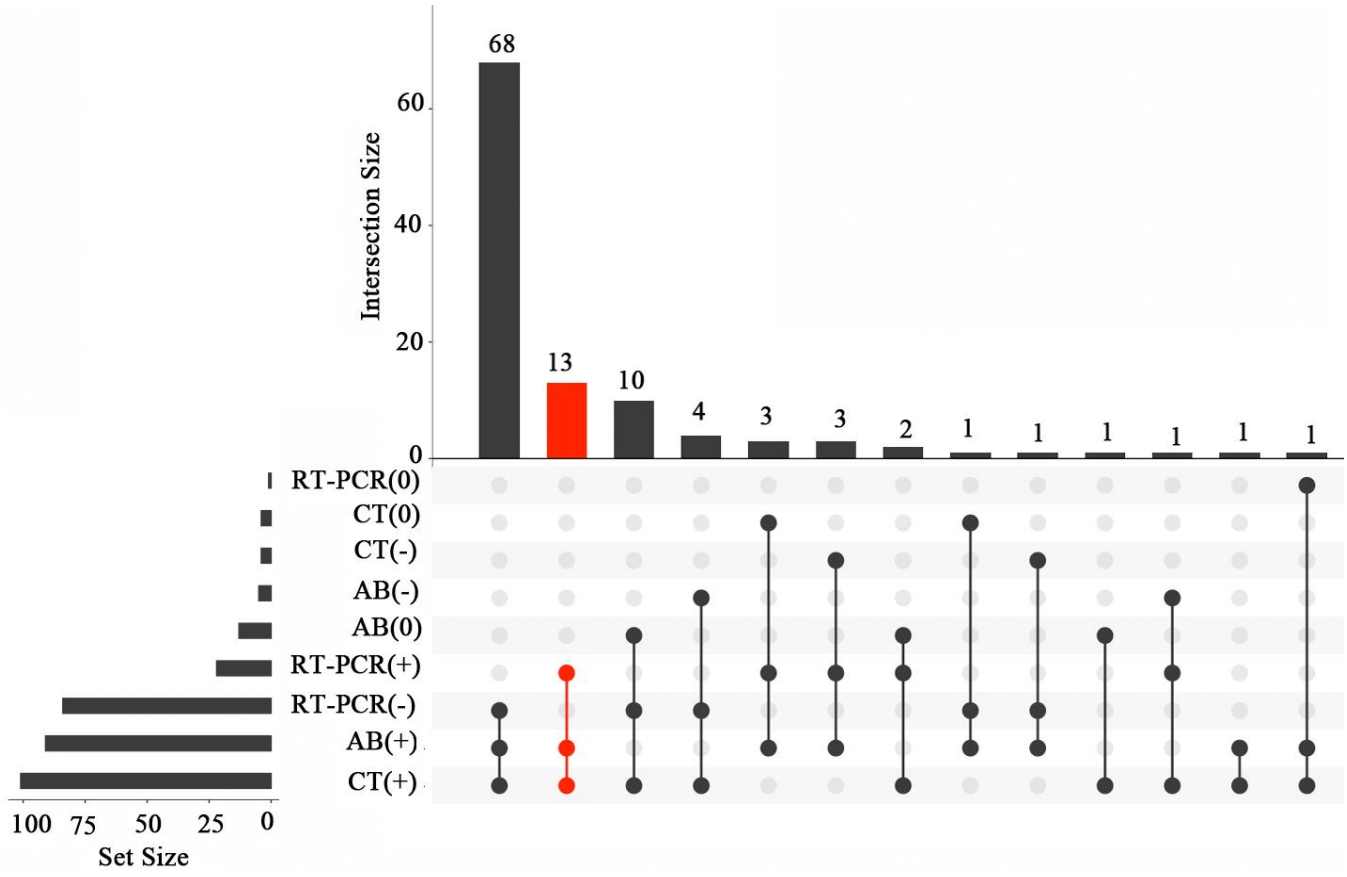

Figure 1. Venn diagram showing the laboratory parameters and CT findings in COVID-19 patients. AB (+), positive antibody assay; AB (-), negative antibody assay; AB (0), antibody assay was not performed; RT-PCR (+), positive RT-PCR assay; RT-PCR (-), negative RT-PCR assay; RT-PCR (0), RT-PCR assay was not performed; CT (+), positive CT diagnosis; CT (-), negative CT diagnosis; CT (0), CT was not performed.

Table 2. Examinations and clinical symptoms of patients with COVID-19.

	All patients	Initial respiratory symptoms[a]		Initial SpO2 value (%)		Supplemental O_2		Respiratory symptoms after O_2 supplement		SpO2 value after O_2 supplement (%)		Respiratory symptoms without O_2 supplement		SpO2 value without O_2 supplement (%)	
		Yes	No	≥94	<94	Yes	No	Yes	No	≥94	<94	Yes	No	≥94	<94
Antibody assay[b]															
Positive	91(83.5)	82(75.3)	9(8.2)	68(62.4)	23(21.1)	82(75.3)	9(8.2)	24(22.0)	58(53.2)	82(75.3)	0(0)	1(0.9)	8(7.3)	9(8.2)	0(0)
Negative	5(4.6)	3(2.8)	2(1.8)	3(2.8)	2(1.8)	5(4.6)	0(0)	1(0.9)	4(3.7)	5(4.6)	0(0)	0(0)	0(0)	0(0)	0(0)
Not performed	13(11.9)	10(9.1)	3(2.8)	7(6.4)	6(5.5)	13(11.9)	0(0)	6(5.5)	7(6.4)	13(11.9)	0(0)	0(0)	0(0)	0(0)	0(0)
RT-PCR assay[c]															
Positive	24(22.0)	22(20.2)	2(1.8)	17(15.5)	7(6.4)	23(21.1)	1(0.9)	12(11.0)	11(10.1)	23(21.1)	0(0)	0(0)	1(0.9)	1(0.9)	0(0)
Negative	84(77.1)	72(66.1)	12(11.0)	60(55.0)	24(22.1)	76(69.7)	8(7.3)	19(17.4)	57(52.3)	76(69.7)	0(0)	1(0.9)	7(6.4)	8(7.3)	0(0)
Not performed	1(0.9)	1(0.9)	0(0)	1(0.9)	0(0)	1(0.9)	0(0)	0(0)	1(0.9)	1(0.9)	0(0)	0(0)	0(0)	0(0)	0(0)
CT diagnosis															
Positive	101(92.6)	88(80.7)	13(11.9)	72(66.1)	29(26.6)	93(85.3)	8(7.3)	27(24.8)	66(60.6)	93(85.3)	0(0)	1(0.9)	7(6.4)	8(7.3)	0(0)
Negative	4(3.7)	3(2.8)	1(0.9)	3(2.8)	1(0.9)	3(2.8)	1(0.9)	2(1.8)	1(0.9)	3(2.8)	0(0)	0(0)	1(0.9)	1(0.9)	0(0)
Not performed	4(3.7)	4(3.7)	0(0)	3(2.8)	1(0.9)	4(3.7)	0(0)	2(1.8)	2(1.8)	4(3.7)	0(0)	0(0)	0(0)	0(0)	0(0)

Data are n (%), unless otherwise specified. Abbreviations: COVID-19, coronavirus disease 2019; SpO2, Percutaneous oxygen saturation; O_2, oxygen.

[a] including cough, sore throat, short of breath, chest tightness, expectoration and dyspnea; [b]Anti-SARS-CoV-2 antibody assay; [c]SARS-CoV-2 RT-PCR assay.

There are several limitations to this study. First, the patient cohort consisted of only 109 cases, but the patients' characteristics were similar to those in previous studies [1, 6, 18]. Second, we did not test for hematological indicators of heart, liver, and kidney function. Additionally, we did not assess for important observation indexes, such as clinical outcomes, medication plans, living conditions, and less common symptoms, which might be vital for clinical decision making and outcome prediction.

In conclusion, our findings suggest that oxygen therapy plays an important role in the treatment of COVID-19 patients. Old age and underlying diseases are the main risk factors of SARS-CoV-2 infection; therefore, the elderly and individuals with chronic diseases should be closely monitored after contracting the virus. Most COVID-19 patients have no fever; hence, thorough clinical examination, CT, and laboratory examination are pivotal for COVID-19 diagnosis. Additionally, the detection rate of CT is superior to anti-SARS-CoV-2 antibody testing and SARS-CoV-2 RT-PCR; thus, CT imaging should be implemented in the clinical practice to confirm SARS-CoV-2 infection.

MATERIALS AND METHODS

Study design and participants

In this study, we analyzed data from 109 COVID-19 patients admitted to the Wuhan Union Hospital in Hubei, China, from February 13, 2020, to February 29, 2020, according to the WHO Interim Guidelines [20]. The study was approved by the Ethics Committee of Wuhan Union Hospital. Informed consent was provided by all patients.

Data collection

We collected the following data from 109 patients diagnosed with SARS-CoV-2 infection: age, gender, respiratory symptoms (fever, cough, dyspnea, chest tightness, sore throat), vital signs at admission (temperature, heart rate, respiratory rate, SpO_2), chronic medical history (chronic heart disease, coronary heart disease, hypertension, hyperlipidemia, diabetes), treatment (O_2 therapy), respiratory symptoms after treatment (fever, cough, dyspnea, chest tightness, sore throat), SpO_2 after treatment, presence of anti-SARS-CoV-2 antibodies, SARS-CoV-2 RT-PCR assay results, and CT findings. Continuous variables were expressed as medians and ranges, whereas categorical variables were expressed as numbers and percentages (%).

Statistical analysis

Differences among groups were analyzed using Fisher's exact test, χ^2 test, univariate analysis, and multivariate analysis according to the type of data. Statistical analyses were conducted using GraphPad Prism 8.0 and TBtools software. *P*-values <0.05 were considered statistically significant.

Abbreviations

COVID-19: coronavirus disease 2019; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; CT: computed tomography; O_2: oxygen; RT-PCR: real-time quantitative polymerase chain reaction; MERS: Middle East respiratory syndrome; SpO_2: percutaneous oxygen saturation.

AUTHOR CONTRIBUTIONS

Lina Liu and Jing Yao collected the data. Yanfang Liu, Ye Wang, and Xinyang Du summarized and analyzed all data. Yanfang Liu and Lina Liu drafted the manuscript. Jing Yao and Hong Ma revised the final manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

2. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

3. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

4. WHO. Coronavirus disease (COVID-19) outbreak situation. 2020. https://www.who.int/emergencies/diseases/novel-coronavirus-2019.

5. Ye G, Pan Z, Pan Y, Deng Q, Chen L, Li J, Li Y, Wang X. Clinical characteristics of severe acute respiratory syndrome coronavirus 2 reactivation. J Infect. 2020; 80:e14–17. https://doi.org/10.1016/j.jinf.2020.03.001 PMID:32171867

6. Xu X, Yu C, Qu J, Zhang L, Jiang S, Huang D, Chen B, Zhang Z, Guan W, Ling Z, Jiang R, Hu T, Ding Y, et al. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2. Eur J Nucl Med Mol Imaging. 2020; 47:1275–80. https://doi.org/10.1007/s00259-020-04735-9 PMID:32107577

7. Zheng J. SARS-CoV-2: an emerging coronavirus that causes a global threat. Int J Biol Sci. 2020; 16:1678–85. https://doi.org/10.7150/ijbs.45053 PMID:32226285

8. Lo IL, Lio CF, Cheong HH, Lei CI, Cheong TH, Zhong X, Tian Y, Sin NN. Evaluation of SARS-CoV-2 RNA shedding in clinical specimens and clinical characteristics of 10 patients with COVID-19 in macau. Int J Biol Sci. 2020; 16:1698–707. https://doi.org/10.7150/ijbs.45357 PMID:32226287

9. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

10. Young BE, Ong SW, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, et al, and Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. JAMA. 2020; 323:1488–94. https://doi.org/10.1001/jama.2020.3204 PMID:32125362

11. Park SE. Epidemiology, virology, and clinical features of severe acute respiratory syndrome -coronavirus-2 (SARS-CoV-2; coronavirus disease-19). Clin Exp Pediatr. 2020; 63:119–24. https://doi.org/10.3345/cep.2020.00493 PMID:32252141

12. Fung SY, Yuen KS, Ye ZW, Chan CP, Jin DY. A tug-of-war between severe acute respiratory syndrome coronavirus 2 and host antiviral defence: lessons from

other pathogenic viruses. Emerg Microbes Infect. 2020; 9:558–70. https://doi.org/10.1080/22221751.2020.1736644 PMID:32172672

13. Lai CC, Shih TP, Ko WC, Tang HJ, Hsueh PR. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges. Int J Antimicrob Agents. 2020; 55:105924. https://doi.org/10.1016/j.ijantimicag.2020.105924 PMID:32081636

14. Ghinai I, McPherson TD, Hunter JC, Kirking HL, Christiansen D, Joshi K, Rubin R, Morales-Estrada S, Black SR, Pacilli M, Fricchione MJ, Chugh RK, Walblay KA, et al, and Illinois COVID-19 Investigation Team. First known person-to-person transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the USA. Lancet. 2020; 395:1137–44. https://doi.org/10.1016/S0140-6736(20)30607-3 PMID:32178768

15. Goyal P, Choi JJ, Pinheiro LC, Schenck EJ, Chen R, Jabri A, Satlin MJ, Campion TR Jr, Nahid M, Ringel JB, Hoffman KL, Alshak MN, Li HA, et al. Clinical characteristics of covid-19 in new york city. N Engl J Med. 2020; 382:2372–74. https://doi.org/10.1056/NEJMc2010419 PMID:32302078

16. Hu Z, Song C, Xu C, Jin G, Chen Y, Xu X, Ma H, Chen W, Lin Y, Zheng Y, Wang J, Hu Z, Yi Y, Shen H. Clinical characteristics of 24 asymptomatic infections with COVID-19 screened among close contacts in nanjing, China. Sci China Life Sci. 2020; 63:706–11. https://doi.org/10.1007/s11427-020-1661-4 PMID:32146694

17. CDC. Groups at Higher Risk for Severe Illness. 2020. https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/

18. Yu J, Ouyang W, Chua ML, Xie C. SARS-CoV-2 transmission in patients with cancer at a tertiary care hospital in Wuhan, China. JAMA Oncol. 2020; 6:1108–10. https://doi.org/10.1001/jamaoncol.2020.0980 PMID:32211820

19. Pan Y, Long L, Zhang D, Yuan T, Cui S, Yang P, Wang Q, Ren S. Potential false-negative nucleic acid testing results for severe acute respiratory syndrome coronavirus 2 from thermal inactivation of samples with low viral loads. Clin Chem. 2020; 66:794–801. https://doi.org/10.1093/clinchem/hvaa091 PMID:32246822

20. WHO. Clinical management of severe acute respiratory infection when COVID-19 is suspected. 2020; https://www.who.int/publications-detail/clinical-management-of-severe-acute-respiratory-infection-when-novel-coronavirus-(ncov)-infection-is-suspected.

SUPPLEMENTARY MATERIALS

Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 1, 2.

Supplementary Table 1. Demographics characteristics of patients with COVID-19.

Supplementary Table 2. Clinical features of patients with COVID-19.

Supplementary Table 3. Laboratory examination and CT diagnosis of patients with COVID-19.

	All patients	Antibody assay positive	Antibody assay negative	Antibody assay not performed	RT-PCR assay positive	RT-PCR assay negative	RT-PCR assay not performed	CT diagnosis positive	CT diagnosis negative	CT diagnosis not performed
Antibody assay[a]										
Positive	91(83.5)	91(83.5)	0(0)	0(0)	20(18.3)	70(64.3)	1(0.9)	83(76.1)	4(3.7)	4(3.7)
Negative	5(4.6)	0(0)	5(4.6)	0(0)	1(0.9)	4(3.7)	0(0)	5(4.6)	0(0)	0(0)
Not performed	13(11.9)	0(0)	0(0)	13(11.9)	3(2.8)	10(9.1)	0(0)	13(11.9)	0(0)	0(0)
RT-PCR assay[b]										
Positive	24(22.0)	20(18.3)	1(0.9)	3(2.8)	24(22.0)	0(0)	0(0)	18(16.5)	3(2.8)	3(2.8)
Negative	84(77.1)	70(64.3)	4(3.7)	10(9.1)	0(0)	84(77.1)	0(0)	82(75.2)	1(0.9)	1(0.9)
Not performed	1(0.9)	1(0.9)	0(0)	0(0)	0(0)	0(0)	1(0.9)	1(0.9)	0(0)	0(0)
CT diagnosis										
Positive	101(92.6)	83(76.1)	5(4.6)	13(11.9)	18(16.5)	82(75.2)	1(0.9)	101(92.6)	0(0)	0(0)
Negative	4(3.7)	4(3.7)	0(0)	0(0)	3(2.8)	1(0.9)	0(0)	0(0)	4(3.7)	0(0)
Not performed	4(3.7)	4(3.7)	0(0)	0(0)	3(2.8)	1(0.9)	0(0)	0(0)	0(0)	4(3.7)

Data are n (%), unless otherwise specified. Abbreviations: COVID-19, coronavirus disease 2019; CT, computed tomography; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-PCR, real-time quantitative polymerase chain reaction. [a]Anti-SARS-CoV-2 antibody assay; [b]SARS-CoV-2 RT-PCR assay.

Research Paper

COVID-19: a probable role of the anticoagulant Protein S in managing COVID-19-associated coagulopathy

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ABSTRACT

The COVID-19 pandemic has caused monumental mortality, and there are still no adequate therapies. Most severely ill COVID-19 patients manifest a hyperactivated immune response, instigated by interleukin 6 (IL6) that triggers a so called "cytokine storm" and coagulopathy. Hypoxia is also associated with COVID-19. So far overlooked is the fact that both IL6 and hypoxia depress the abundance of a key anticoagulant, Protein S. We speculate that the IL6-driven cytokine explosion plus hypoxemia causes a severe drop in Protein S level that exacerbates the thrombotic risk in COVID-19 patients. Here we highlight a mechanism by which the IL6-hypoxia curse causes a deadly hypercoagulable state in COVID-19 patients, and we suggest a path to therapy.

INTRODUCTION

The menacing SARS-CoV2 virus has caused a pandemic with over 6.9 million cases and around 400,000 deaths. As of to date (07/11/2020), there are more than 3.2 million confirmed cases and ~134,729 deaths in the U.S. Clinical features of patients admitted to the hospital with the viral disease COVID-19 are bilateral pneumonia, systemic inflammation, endothelial dysfunction, coagulation activation, acute respiratory distress syndrome, and multi-organ failure. Signs of myocardial injury are also observed in at least one quarter of severe cases. Although the lung is the main target organ, the virus can infect other tissues (small intestine, testis, kidneys, heart, thyroid, adipose tissue, colon, liver, bladder, adrenal gland [1]).

The infection risk of SARS-CoV2 has no remarkable correlation with age because the expression of the virus receptor ACE2 does not vary much between young and old; however, mortality is significantly higher in older people compared with the young, (Table 1). SARS-CoV2 infection was found to reduce the expression of ACE2 in lungs, leading to a renin-angiotensin system (RAS) dysfunction. This RAS dysfunction, in turn, would enhance inflammation and vascular permeability in the airways [2].

However, unlike the SARS-CoV pandemic in 2003, COVID-19 is not simply a disease of the upper respiratory tract. COVID-19 patients experience hypercoagulability and increased risk of venous thromboembolism (Table 2). These thrombotic complications have been referred to as *thrombo-inflammation* or *COVID-19-associated coagulopathy* [3–7]. Moreover, several reports indicate that hypercoagulability, as measured by the D-Dimer levels, is present mostly in critically ill and deceased patients (Table 2). In addition to blood clots of all sizes throughout the body, doctors who treat coronavirus patients report a range of other odd and frightening syndromes, such as kidney failure, cardiac inflammation, and immune complications. These syndromes appear to arise from a SARS-CoV2 virus-induced local inflammatory response.

Table 1. Different parameters of COVID-19 patients.

Place	Time and Date	No of patient	Sex		Age	Mortality
			Male	Female		
Netherlands[42]	7th March - 5th April, 2020	184	139	45	Average : 64	23
Lombardy region of Italy[43]	20th February -18th March, 2020	1591	1304	287	Median : 63	405
Italy[44]	Until 15th March, 2020	22512	13462	9050	Median : 64	1625
Zhongnan Hospital of Wuhan University in Wuhan, China[4]	1st January to 13th March, 2020	449	268	181	Average : 65.1	134
Fatal Cases of COVID-19 from Wuhan China[20]	9th January-15th February, 2020	85	62	23	Median: 65.8	All
Wuhan Jin Yin-tan Hospital, Wuhan, China[45]	Late December, 2019-26th January, 2020	52	36	17	Average: 59.7	32

Table 2. Studies which indicate that hypercoagulability (supra-physiological levels of D-dimer), is almost always associated with disease severity and mortality of COVID-19.

Study	Sample size	Mean D-dimer (<0.5 μg/ml)	p-values	Comment
Tang et al, Feb 2020,[34]	Survivors (162) Non-survivors (21)	0.6 2.12	<0.001	Disseminated intravascular coagulation (DIC) was found in most deaths
Han et al, Mar 2020, [33]	Ordinary patient (49) Critical (10)	2.14 ±2.88 20.04 ± 32.39	<0.001 <0.05	Huge increase in D-dimer in critically ill COVID patients
Wang et al, Mar 2020, [46]	ICU (36) Non-ICU (102)	4.14 1.66	<0.001	In the non-survivors, D-dimer increased continuously
Zhang et al, April 2020, [47]	Ordinary (276) Severe (67)	0.41 4.76	<0.001	12 non-survivors had D-dimer values greater than 2.0
Spiezia et al, April 2020, [48]	ICU (22)	5.343 ±2.099	<0.0001	All ICU patients with acute respiratory failure showed severe hypercoagulability, one patient with the most hypercoagulable state died.
Ranucci et al, April 2020, [35]	Total (16)	3.5	0.017	Seven patients died of hypoxia and multi-organ failure
Tang et al, May 2020, [49]	Survivors (315) Non-survivors (134)	1.47 4.7	<0.001	30 of the non survivors died even after treated with low molecular weight heparin

Because of lung involvement, most COVID-19 patients have exceedingly low blood oxygen levels, but, inexplicably, some of these hypoxic patients hardly gasp for breath. Alarmingly, these individuals are subject, without warning, to sudden shortness of breath and massive pulmonary embolism [8]. Note that bleeding is rare in the current onset of the disease.

Our past studies [9–13] with the natural anticoagulant Protein S illuminated our understanding about the significance of Protein S -Factor IXa interaction in hemostasis. Further, we identified a critical role of Protein S in regulating hypoxia and associated thrombotic complications [9].

The overarching goal of this article is to propose a strategy to better control the hypoxemia associated hypercoagulability in severe COVID-19 patients.

Inflammation, coagulation and hypoxia

Inflammation, as a part of the innate immunity response to an infection, triggers activation of coagulation pathways. Activation of coagulation influenced by

inflammation, in turn, can modulate the inflammatory response. The coordinated activation of both coagulation and inflammation during a severe infection is a well-recognized phenomenon known as thrombo-inflammation. Thrombo-inflammation is associated with microvascular thrombosis, hypoxemic respiratory failure, and, in extreme cases, it may lead to death due to development of multiple organ dysfunction syndrome (MODS) [14]. A disproportionate inflammatory response to SARS-CoV2 is associated with exorbitant circulating levels of inflammatory cytokines, which is thought to be a major cause of disease severity and death [15].

The main mediators of inflammation-activated coagulation are the pro-inflammatory cytokines [16]. In severe sepsis, the pro-inflammatory cytokines stimulate mononuclear cells expressing more and more tissue factor that initiate the coagulation pathways. Interleukin 6 (IL-6) is the most important cytokine that influences the expression of tissue factor which activates coagulation.

Thrombo-inflammation – occurs by overproduction of early response proinflammatory cytokines (TNFα, IL-6, and IL-1β) that create a "cytokine storm" [4, 15, 17–22]. This cytokine explosion leads to increased risk of vascular hyperpermeability, multi-organ failure, and eventually death when high cytokine concentrations persist [23, 24]. The inflammatory effects of cytokines also activate vascular endothelial cells and cause endothelial injury with resultant prothrombotic properties [25]. Independent transcriptome datasets from infection models revealed that IL6 is the major cytokine differentially expressed after infection with SARS- CoV2 [26–30].

Autopsies revealed microthrombi in lungs and other organs with associated foci of hemorrhage [31]. Such observations suggest that severe endothelial dysfunction, driven by the cytokine storm and associated hypoxemia, lead to disseminated intravascular coagulation and thromboembolic complications. Importantly, development of local hypoxia will progressively intensify endothelial cell disruption, tissue factor expression, and activation of the coagulation cascade, thereby establishing a deadly positive thrombo-inflammatory feedback loop with thrombosis and hemorrhage occurring in the small vessels of the lungs.

In summary, severe hypoxia is now considered associated with gravely ill COVID-19 patients, and IL6 is upregulated in COVID-19 and promotes *cis* and *trans* signaling to produce a cytokine storm [32]. Further, it is reasonable to conclude that subtle clotting begins early in the lungs, perhaps due to an inflammatory reaction in their fine web of blood vessels, which then sets off a cascade of proteins that prompt blood to clot and prevent proper oxygenation. Blood clots are clearly a major contributor to COVID-19 disease severity and mortality.

Crosstalk between thrombotic complications and inflammation/cytokine storm in SARS-CoV2 infection

Severity of COVID-19 is commonly associated with coagulopathy; disseminated intravascular coagulation (DIC) being the predominant condition along with high venous thromboembolism rates, and pulmonary congestion with microvascular thrombosis [33]. In general, hemostatic system alterations were indicated by prolonged aPTT, elevated platelet count, increased D-dimer level and fibrin degradation product for patients with severe COVID 19 [34]. Fibrin deposition in alveolar and interstitial lung spaces, in addition to microcirculation thrombosis, may exacerbate respiratory symptoms that require prolonged mechanical ventilation, and which are associated with poor prognosis and death. D-dimer levels have been identified as markers of severity of the disease and predictive of mortality [34]. Ranucci et. al. [35] incorporated viscoelastic tests for ICU patients along with the other commonly performed examinations. The test provides information about clot time (CT), clot strength (CS), fibrinogen contribution (FCS), and platelet contribution (PCS) to clot strength. Patient procoagulant profiles were confirmed by increased CS, FCS and PCS. Increased clot strength has been correlated to high fibrinogen level and somewhat to elevated platelet count.

COVID-19 disease severity is also associated with acute lung injury and hypoxemic respiratory failure, the most common cause of death. High levels of cytokines and chemokines associated with T cell depletion, pulmonary inflammation, and extensive lung damage have been documented in individuals who experienced similar viral respiratory diseases such as SARS and MERS. Thus, the wide-spread lung damage associated with this kind of infection may be caused more by an exaggerated immune response than by the virus itself. In addition, supraphysiological levels of IL-6, IL-10 and TNF-α have been found in the sera of severely ill COVID 19 patients [35]. Therefore, all patients with severe COVID-19 should be screened for excessive inflammation by measuring cytokine levels to stratify patients eligible for a specific immunosuppressive treatment [36].

The prevalence of both a cytokine storm and derangement of coagulation in critically ill COVID-19

patients signifies the aforesaid synergy between inflammation and coagulation. A clear association between increased IL-6 and fibrinogen level was reported for a set of COVID 19 ICU patients [35]. Recent guidance from the International Society on Thrombosis and Hemostasis (ISTH) stresses the need for monitoring coagulation parameters for patients who develop sepsis from the infection. The only widely available standard of care in this respect is a prophylactic dose of low molecular weight heparin, which should be considered for all COVID 19 patients (including non-critically ill) with high D-dimer levels, except for patients in whom anticoagulants are not advisable. For patients allergic to heparin, fondaparinux, a synthetic pentasaccharide, is an alternative. Fondaparinux has antithrombotic activity due to anti-thrombin-mediated selective inhibition of FXa. Systematic anticoagulation therapy for hospitalized COVID 19 patients is now routine treatment.

IL6, Hypoxia and Protein S

An overlooked aspect of hypoxia and the IL6-induced cytokine storm is that *both* factors downregulate a key anticoagulant, Protein S [9, 32] (Figure 1). For example, in a population of stroke patients, IL6 was upregulated, and it caused downregulation of Protein S that resulted in venous thrombosis [37]. We demonstrated that hypoxia downregulates Protein S expression in HepG2 cells [9]. Further, we showed that Protein S supplementation in thrombotic mice (mimicking hypoxic niche due to constitutive stabilization of HIF1α) plasma was able to alleviate the thrombotic risk [9]. Notably, addition of Protein S in normal mice plasma reduced thrombin generation as well [9]. These data indicate that Protein S supplementation could be useful in treating thrombotic complications. A substantial number of severe COVID-19 patients manifest both hypoxia and prothrombotic complications [34, 38–40] and we speculate that reduced Protein S level might play a key role in the disease progression of these patients.

Ordinarily, Protein S deficiency is due either to homozygous or heterozygous genetic alteration, and Protein S deficiency can result from various pathological states and diseases. In all cases, Protein S deficiency is associated with a higher risk of venous thrombosis. Because both hypoxia and IL6-induced inflammation depress Protein S abundance, it's reasonable to consider administration of Protein S as an effective therapy in severe Covid19 patients. Indeed, therapeutic heparin has improved the conditions of COVID-19 patients who experienced hypoxia. However, heparin targets FIXa, FXa and thrombin [41] through antithrombin. Therefore, direct administration of Protein S should have a highly specific anticoagulant effect in any thrombotic complications caused by Protein S deficiency. Of course, the possibility of

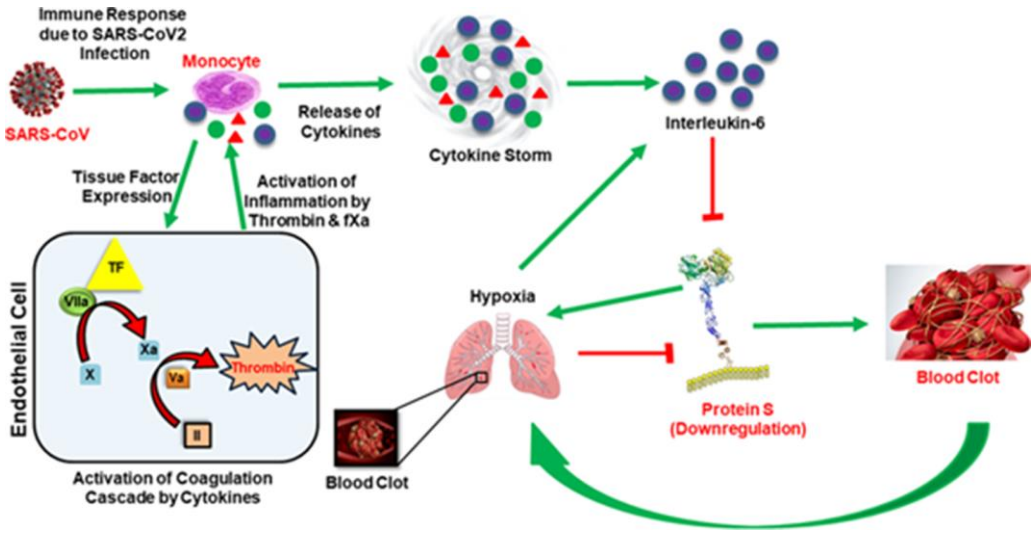

Figure 1. In the presence of the SAR-COV2 virus, early response proinflammatory cytokines (IL-6, TNFα, IL-1β etc.) are induced and activate the coagulation cascade by stimulating tissue factor (TF) expression from monocytes. The presentation of tissue factor leads to the formation of thrombin by the TF-VIIa pathway. Thrombin produces clots, and clots get wedged into arteries in the lungs and cause thrombotic complications and hypoxia. Hypoxia also induces IL-6. Simultaneously, thrombin augments inflammation and accelerates the production of proinflammatory cytokines, termed 'cytokine storm'. Both cytokine storm and hypoxia downregulate Protein S, leading to coagulopathy. Green arrows represent upregulation and red blockage represent downregulation.

bleeding would need attention, but, fortunately, even high doses of heparin have not caused bleeding in COVID-19 patients. Nonetheless, before Protein S administration can be deemed a new therapeutic approach, it is necessary to determine the extent to which Protein S is downregulated in a large cohort of COVID-19 patients. In view of the double curse of hypoxia and IL6, we expect Protein S deficiency to be severe in COVID-patients. However, we acknowledge that testing for safety and efficacy as well as FDA approval would be required before this approach could be implemented.

AUTHOR CONTRIBUTIONS

SC and TS - contributed to writing the first draft, assembling the references, and composing the Figure. SM - provided critical biological input, edited the final Figure and revised the resubmitted manuscript. RM-conceptualized the work and wrote the final draft.

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CONFLICTS OF INTEREST

The author declares no conflicts of interest.

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REFERENCES

1. Li MY, Li L, Zhang Y, Wang XS. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. Infect Dis Poverty. 2020; 9:45. https://doi.org/10.1186/s40249-020-00662-x PMID:32345362

2. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LF. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol. 2020; 20:363–74. https://doi.org/10.1038/s41577-020-0311-8 PMID:32346093

3. Connors JM, Levy JH. Thromboinflammation and the hypercoagulability of COVID-19. J Thromb Haemost. 2020; 18:1559–61. https://doi.org/10.1111/jth.14849 PMID:32302453

4. Bikdeli B, Madhavan MV, Jimenez D, Chuich T, Dreyfus I, Driggin E, Nigoghossian C, Ageno W, Madjid M, Guo Y, Tang LV, Hu Y, Giri J, et al, and Global COVID-19 Thrombosis Collaborative Group, Endorsed by the ISTH, NATF, ESVM, and the IUA, Supported by the ESC Working Group on Pulmonary Circulation and Right Ventricular Function. COVID-19 and thrombotic or thromboembolic disease: implications for prevention, antithrombotic therapy, and follow-up: JACC state-of-the-art review. J Am Coll Cardiol. 2020; 75:2950–73. https://doi.org/10.1016/j.jacc.2020.04.031 PMID:32311448

5. Fogarty H, Townsend L, Ni Cheallaigh C, Bergin C, Martin-Loeches I, Browne P, Bacon CL, Gaule R, Gillett A, Byrne M, Ryan K, O'Connell N, O'Sullivan JM, et al. COVID19 coagulopathy in Caucasian patients. Br J Haematol. 2020; 189:1044–1049. https://doi.org/10.1111/bjh.16749 PMID:32330308

6. Becker RC. COVID-19 update: covid-19-associated coagulopathy. J Thromb Thrombolysis. 2020; 50:54–67. https://doi.org/10.1007/s11239-020-02134-3 PMID:32415579

7. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. Lancet Haematol. 2020; 7:e438–40. https://doi.org/10.1016/S2352-3026(20)30145-9 PMID:32407672

8. Ullah W, Saeed R, Sarwar U, Patel R, Fischman DL. COVID-19 complicated by acute pulmonary embolism and right-sided heart failure. JACC Case Rep. 2020; 2:1379–82. https://doi.org/10.1016/j.jaccas.2020.04.008 PMID:32313884

9. Pilli VS, Datta A, Afreen S, Catalano D, Szabo G, Majumder R. Hypoxia downregulates protein S expression. Blood. 2018; 132:452–55. https://doi.org/10.1182/blood-2018-04-841585 PMID:29784640

10. Chattopadhyay R, Sengupta T, Majumder R. Inhibition of intrinsic xase by protein S: a novel regulatory role of protein S independent of activated protein C. Arterioscler Thromb Vasc Biol. 2012; 32:2387–93. https://doi.org/10.1161/ATVBAHA.112.250928 PMID:22904276

11. Plautz WE, Chattopadhyay R, Goldfeld EI, Samelson-Jones BJ, Pilli VS, Campello E, Datta A, Arruda VR, Simioni P, Majumder R. Padua FIXa resistance to protein S and a potential therapy for hyperactive FIXa. Thromb Res. 2018; 170:133–41. https://doi.org/10.1016/j.thromres.2018.08.018 PMID:30189336

12. Plautz WE, Sekhar Pilli VS, Cooley BC, Chattopadhyay R, Westmark PR, Getz T, Paul D, Bergmeier W, Sheehan JP, Majumder R. Anticoagulant protein S targets the factor IXa heparin-binding exosite to prevent

thrombosis. Arterioscler Thromb Vasc Biol. 2018; 38:816–28.
https://doi.org/10.1161/ATVBAHA.117.310588
PMID:29419409

13. Fricke DR, Chatterjee S, Majumder R. Protein S in preventing thrombosis. Aging (Albany NY). 2019; 11:847–48.
https://doi.org/10.18632/aging.101798
PMID:30683834

14. Zaim S, Chong JH, Sankaranarayanan V, Harky A. COVID-19 and multiorgan response. Curr Probl Cardiol. 2020; 45:100618.
https://doi.org/10.1016/j.cpcardiol.2020.100618
PMID:32439197

15. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, and HLH Across Speciality Collaboration, UK. COVID-19: consider cytokine storm syndromes and immunosuppression. Lancet. 2020; 395:1033–34.
https://doi.org/10.1016/S0140-6736(20)30628-0
PMID:32192578

16. Levi M, van der Poll T. Inflammation and coagulation. Crit Care Med. 2010; 38:S26–34.
https://doi.org/10.1097/CCM.0b013e3181c98d21
PMID:20083910

17. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. Science. 2020; 368:473–74.
https://doi.org/10.1126/science.abb8925
PMID:32303591

18. Panigrahy D, Gilligan MM, Huang S, Gartung A, Cortés-Puch I, Sime PJ, Phipps RP, Serhan CN, Hammock BD. Inflammation resolution: a dual-pronged approach to averting cytokine storms in COVID-19? Cancer Metastasis Rev. 2020; 39:337–40.
https://doi.org/10.1007/s10555-020-09889-4
PMID:32385712

19. Henderson LA, Canna SW, Schulert GS, Volpi S, Lee PY, Kernan KF, Caricchio R, Mahmud S, Hazen MM, Halyabar O, Hoyt KJ, Han J, Grom AA, et al. On the alert for cytokine storm: immunopathology in COVID-19. Arthritis Rheumatol. 2020; 72:1059–63.
https://doi.org/10.1002/art.41285
PMID:32293098

20. Du Y, Tu L, Zhu P, Mu M, Wang R, Yang P, Wang X, Hu C, Ping R, Hu P, Li T, Cao F, Chang C, et al. Clinical features of 85 fatal cases of COVID-19 from Wuhan. A retrospective observational study. Am J Respir Crit Care Med. 2020; 201:1372–79.
https://doi.org/10.1164/rccm.202003-0543OC
PMID:32242738

21. Chau VQ, Oliveros E, Mahmood K, Singhvi A, Lala A, Moss N, Gidwani U, Mancini DM, Pinney SP, Parikh A. The imperfect cytokine storm: severe COVID-19 with ARDS in patient on durable LVAD support. JACC Case Rep. 2020; 2:1315–20.
https://doi.org/10.1016/j.jaccas.2020.04.001
PMID:32292915

22. Chiappelli F, Khakshooy A, Greenberg G. CoViD-19 immunopathology and immunotherapy. Bioinformation. 2020; 16:219–22.
https://doi.org/10.6026/97320630016219
PMID:32308263

23. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. Chest. 1995; 108:1303–14.
https://doi.org/10.1378/chest.108.5.1303
PMID:7587434

24. Magro C, Mulvey JJ, Berlin D, Nuovo G, Salvatore S, Harp J, Baxter-Stoltzfus A, Laurence J. Complement associated microvascular injury and thrombosis in the pathogenesis of severe COVID-19 infection: a report of five cases. Transl Res. 2020; 220:1–13.
https://doi.org/10.1016/j.trsl.2020.04.007
PMID:32299776

25. Iba T, Levy JH. Inflammation and thrombosis: roles of neutrophils, platelets and endothelial cells and their interactions in thrombus formation during sepsis. J Thromb Haemost. 2018; 16:231–41.
https://doi.org/10.1111/jth.13911 PMID:29193703

26. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506.
https://doi.org/10.1016/S0140-6736(20)30183-5
PMID:31986264

27. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, Kritas SK. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. J Biol Regul Homeost Agents. 2020; 34:1.
https://doi.org/10.23812/CONTI-E
PMID:32171193

28. McGonagle D, Sharif K, O'Regan A, Bridgewood C. The role of cytokines including interleukin-6 in COVID-19 induced pneumonia and macrophage activation syndrome-like disease. Autoimmun Rev. 2020; 19:102537.
https://doi.org/10.1016/j.autrev.2020.102537
PMID:32251717

29. Chen X, Zhao B, Qu Y, Chen Y, Xiong J, Feng Y, Men D, Huang Q, Liu Y, Yang B, Ding J, Li F. Detectable serum SARS-CoV-2 viral load (RNAaemia) is closely correlated with drastically elevated interleukin 6 (IL-6) level in

critically ill COVID-19 patients. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa449 PMID:32301997

30. Ulhaq ZS, Soraya GV. Interleukin-6 as a potential biomarker of COVID-19 progression. Med Mal Infect. 2020; 50:382–83. https://doi.org/10.1016/j.medmal.2020.04.002 PMID:32259560

31. Danzi GB, Loffi M, Galeazzi G, Gherbesi E. Acute pulmonary embolism and COVID-19 pneumonia: a random association? Eur Heart J. 2020; 41:1858. https://doi.org/10.1093/eurheartj/ehaa254 PMID:32227120

32. de Wolf CJ, Cupers RM, Bertina RM, Vos HL. Interleukin-6 induction of protein s is regulated through signal transducer and activator of transcription 3. Arterioscler Thromb Vasc Biol. 2006; 26:2168–74. https://doi.org/10.1161/01.ATV.0000236202.39165.eb PMID:16840717

33. Han H, Yang L, Liu R, Liu F, Wu KL, Li J, Liu XH, Zhu CL. Prominent changes in blood coagulation of patients with SARS-CoV-2 infection. Clin Chem Lab Med. 2020; 58:1116–20. https://doi.org/10.1515/cclm-2020-0188 PMID:32172226

34. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. J Thromb Haemost. 2020; 18:844–47. https://doi.org/10.1111/jth.14768 PMID:32073213

35. Ranucci M, Ballotta A, Di Dedda U, Bayshnikova E, Dei Poli M, Resta M, Falco M, Albano G, Menicanti L. The procoagulant pattern of patients with COVID-19 acute respiratory distress syndrome. J Thromb Haemost. 2020; 18:1747–51. https://doi.org/10.1111/jth.14854 PMID:32302448

36. Sarzi-Puttini P, Giorgi V, Sirotti S, Marotto D, Ardizzone S, Rizzardini G, Antinori S, Galli M. COVID-19, cytokines and immunosuppression: what can we learn from severe acute respiratory syndrome? Clin Exp Rheumatol. 2020; 38:337–42. PMID:32202240

37. Vila N, Reverter JC, Yagüe J, Chamorro A. Interaction between interleukin-6 and the natural anticoagulant system in acute stroke. J Interferon Cytokine Res. 2000; 20:325–29. https://doi.org/10.1089/107999000312478 PMID:10762081

38. Marongiu F, Grandone E, Barcellona D. Pulmonary thrombosis in 2019-nCoV pneumonia? J Thromb Haemost. 2020; 18:1511–13. https://doi.org/10.1111/jth.14818 PMID:32293083

39. Tang N. Response to 'Pulmonary thrombosis in 2019-nCoV pneumonia?'. J Thromb Haemost. 2020; 18:1513–14. https://doi.org/10.1111/jth.14843 PMID:32291906

40. Arachchillage DR, Laffan M. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. J Thromb Haemost. 2020; 18:1233–34. https://doi.org/10.1111/jth.14820 PMID:32291954

41. Hirsh J, Anand SS, Halperin JL, Fuster V. Mechanism of action and pharmacology of unfractionated heparin. Arterioscler Thromb Vasc Biol. 2001; 21:1094–96. https://doi.org/10.1161/hq0701.093686 PMID:11451734

42. Klok FA, Kruip MJ, van der Meer NJ, Arbous MS, Gommers DA, Kant KM, Kaptein FH, van Paassen J, Stals MA, Huisman MV, Endeman H. Incidence of thrombotic complications in critically ill ICU patients with COVID-19. Thromb Res. 2020; 191:145–147. https://doi.org/10.1016/j.thromres.2020.04.013 PMID:32291094

43. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, Cereda D, Coluccello A, Foti G, Fumagalli R, Iotti G, Latronico N, Lorini L, et al, and COVID-19 Lombardy ICU Network. Baseline characteristics and outcomes of 1591 patients infected with SARS-CoV-2 admitted to ICUs of the lombardy region, Italy. JAMA. 2020; 323:1574–81. https://doi.org/10.1001/jama.2020.5394 PMID:32250385

44. Livingston E, Bucher K. Coronavirus disease 2019 (COVID-19) in Italy. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.4344 PMID:32181795

45. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

46. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

47. Zhang L, Yan X, Fan Q, Liu H, Liu X, Liu Z, Zhang Z. D-

dimer levels on admission to predict in-hospital mortality in patients with covid-19. J Thromb Haemost. 2020; 18:1324–29. https://doi.org/10.1111/jth.14859 PMID:32306492

48. Spiezia L, Boscolo A, Poletto F, Cerruti L, Tiberio I, Campello E, Navalesi P, Simioni P. COVID-19-related severe hypercoagulability in patients admitted to intensive care unit for acute respiratory failure. Thromb Haemost. 2020; 120:998–1000. https://doi.org/10.1055/s-0040-1710018 PMID:32316063

49. Tang N, Bai H, Chen X, Gong J, Li D, Sun Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. J Thromb Haemost. 2020; 18:1094–99. https://doi.org/10.1111/jth.14817 PMID:32220112

Letter to the Editors

A newborn with normal IgM and elevated IgG antibodies born to an asymptomatic infection mother with COVID-19

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ABSTRACT

Pregnant women are susceptible population of COVID-19 which are more likely to have complications and even progress to severe illness. Pregnancy with COVID-19 and neonates are rarely reported. We report a newborn with normal IgM and elevated IgG antibodies born to an asymptomatic infection mother with COVID-19. We assessed whether there was intrauterine vertical transmission potential of COVID-19.

DEAR EDITOR

We reported a newborn with normal IgM and elevated IgG antibodies born to an asymptomatic infection mother with coronavirus disease 2019 (COVID-19). In our present case, the mother of the neonate was a 30-year-old pregnant woman. There were no confirmed or suspected cases of COVID-19 in her family. She denied having a history of exposure to COVID-19 patients. She was pregnant for the first time. She claimed that she had never had syphilis, hepatitis b, AIDS and other infectious diseases.

March 4, 2020, the gestation pregnant woman was 39 weeks pregnant. Color ultrasound indicated that the fetus was in the breech position with the umbilical cord around the neck. At 02:05 on March 6, 2020, the pregnant woman went to Wuhan Central Hospital for treatment due to excessive amniotic fluid and umbilical cord around the neck. There were no typical symptoms of COVID-19, such as fever and cough, in this pregnant woman. Thoracic computerized tomography scan revealed no abnormality. The nucleic acid test of pharyngeal swab showed positive, and the results of serum IgM and IgG antibody (colloidal gold method) were weak positive and strong positive, respectively, suggesting that the pregnant woman might be an asymptomatic infection case of COVID-19. Blood tests showed lymphocytes (0.82×10^9/L, normal: $1.1\text{-}3.2\times10^9$/L) reduced. She was hospitalized for suspected viral pneumonia.

On admission, her body temperature was 36.4°C and her blood pressure was 108/65 mmHg, with respiratory rate of 20 breaths per minute, pulse of 76 beats per minute. Cardiopulmonary function was normal, and there was no edema in the lower limbs. No intrauterine distress was presented throughout the pregnancy. Amniotic fluid slant overloaded, without amniotic fluid pollution. Fetal heart monitoring showed no abnormalities, and the fetal heart rate was 140 bpm. Emergency cesarean section was operated for pregnant women. The pregnant woman wore an N95 mask throughout the operation, without cough or produce sputum.

At 14:00 on March 6, 2020 a baby girl was born, weighted 3,460g. Apgar scores at 1 and 5 minutes were 9 and 10, respectively. The baby did not get groan, fever, cough, and vomit. The baby had a ruddy face and a powerful cry. Since there was no isolation ward in the neonatal department of Wuhan Central Hospital, the neonate was transferred to COVID-19 children's designated hospital-Wuhan Children's Hospital immediately after 3 hours of birth. The newborn could eat normal breast milk. The newborn's mental response was good, with blood oxygen saturation maintaining more than 92%. Her body temperature and body length were 36.9°C and 50 cm, with respiratory rate of 36 breaths per minute, pulse of 135 beats per minute.

Laboratory reports of this infant were negative, including toxoplasma, herpes simplex virus 1/2, cytomegalovirus (CMV), and rubella virus. Neutrophils percentage (69.5%, normal: 31% ~ 52%), basophilic cells percentage (0.70%, normal: 0% ~ 0.6%), neutrophils total (13.45%, normal: 3.9% ~ 9.4%) were all increased. Liver dysfunction (AST 92U/L, normal: ≤41 U/L), creatine kinase (189U/L, normal: 30 ~ 170 U/L), creatine kinase isoenzyme MB (60U/L, normal: 0 ~ 24U/L) levels increased. Procalcitonin increased (0.880ng/ml, normal: ≤0.05ng/ml). IL-6 (49.00pg/ml, normal: 0 ~ 20.9pg/ml) and IL-10 (6.28pg/ml, normal: 0 ~ 5.9pg/ml) increased. Renal function and electrolytes were normal. Figure 1A Chest X-ray showed enhanced lung veins, reticular and patchy shadows, and no abnormalities in heart and palate (image). She was closely monitored in isolation, treated with a nourishing cardiac muscle and a spray of interferon. Intravenous injection of penicillin G (15wu q.d, intravenous bolus) and vitamin K1 (1mg q.d, intravenously) were used as antibiotics and to prevent coagulation disorders.

March 7, 2020 the second day after the surgery, the newborn was in good condition and the mother's vital signs were stable. Baby was closely monitored and given 30ml of formula every three hours.

From March 7 to March 12, 2020 the newborn's vital signs were stable, the blood oxygen saturation maintaining above 90%, and there was no apnea or vomit. On March 9, 2020 the nucleic acid test of neonatal COVID-19 pharyngeal swab was negative, however, and serum COVID-19 IgM and IgG antibodies were normal and strong positive, respectively. The blood routine, liver function, calcitonin, and creatine kinase levels all returned to normal. Chest X-ray of neonate showed a few flaky shadow, with no abnormality in heart and palate. Compared with the chest X-ray on March 6, the Chest X-ray Figure 1B of March 12, 2020 revealed that most of the lung lesions were absorbed. She did not receive any special treatment since March 12. On March 17, laboratory tests and chest radiographs were normal, the nucleic acid test of neonatal COVID-19 pharyngeal swab and serum IgM were both negative. The newborn was discharged from hospital on March 18, 2020.

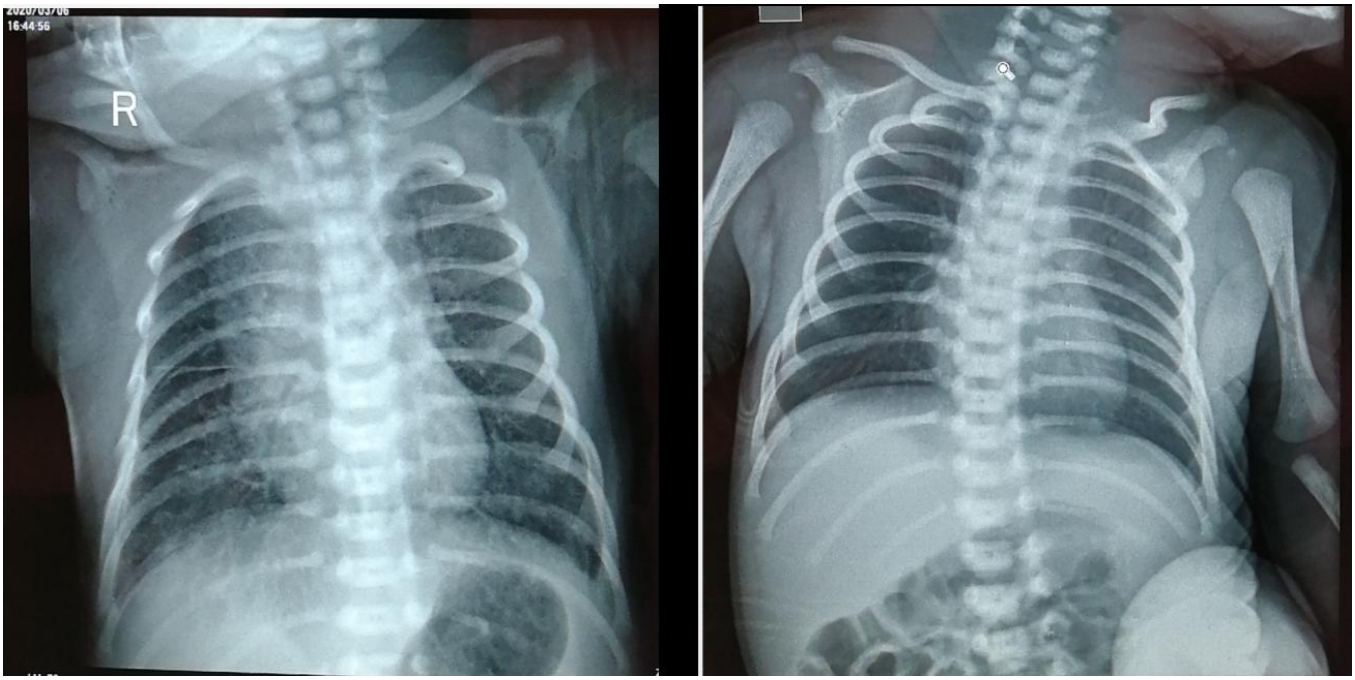

Figure 1. Chest X-ray of neonate on March 6th, 2o20 (**A**) and March 12th, 2020 (**B**).

June 17, 2020 about one hundred days after the neonate was born, we detected the serum COVID-19 IgM and IgG antibodies of mother and infant again. The serum COVID-19 IgM and IgG antibodies of mother were still negative and positive, while the IgG antibody of infant decreased rapidly and changed into negative.

Since December 2019, pneumonia caused by SARS-CoV-2 has become a highly contagious disease. As of April 27, 2020, a total of 2,878,196 COVID–19 cases and 198,668 related deaths have been confirmed [1]. Since COVID-19 was a brand new infectious disease and the immunological detection reagent has just been developed, there were few reports of COVID-19 vertical transmission tracing in pregnant women. Here, we reported a newborn with normal IgM and elevated IgG antibodies born to an asymptomatic infection mother with COVID-19. The viral nucleic acid of the pharyngeal swab in newborn was negative. The pathogenic test found that the serum of IgM of the newborn was normal, and IgG was strongly positive. In general, after the body infecting with pathogenic microorganisms, the immune system carries out immune defense against the virus and produces specific antibodies. Specific IgM may indicate a current or recent infection. IgG is the main antibody produced in the immune response, indicating that the disease has entered the recovery period or the presence of previous infection [2–3].

When the infant was born, the level of IgG antibody in her serum was similar to that of her mother, both strong positive. However, the infant IgG antibody decreased rapidly and turned negative after about one hundred days, while the maternal IgG antibody remains at a high level. According to the examination results of mother and neonate, we suspected that, on the one hand, the neonate might acquire the IgG antibody from mother via placenta. The infant did not produce IgG antibody, therefore, the level of IgG antibody decreased remarkably at time passed. On the other hand, the mother might expose to small numbers of virus before, and was an asymptomatic infection case of COVID-19, thus, the neonate might be in the recovery period of COVID-19 at present. Because of the children's immune systems were underdeveloped, the level of IgG antibody in infant reduced rapidly.

There were several limitations in this study. 1) Lack of nucleic acid detection results in breast milk and placenta; 2) Our present report include the single case, more information was need to confirm the observation.

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CONFLICTS OF INTEREST

All the authors declare no conflicts of interest.

CONSENT FOR PUBLICATION

The patient and his parents all agreed to publish this study via written consent. The consent was obtained from a parent of legal guardian of the patient.

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REFERENCES

1. Coronavirus disease 2019 (COVID-19) Situation Report – 98. https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200427-sitrep-98-covid-19.pdf?sfvrsn=90323472_4

2. Kohler PF, Farr RS. Elevation of cord over maternal IgG immunoglobulin: evidence for an active placental IgG transport. Nature. 1966; 210:1070–71. https://doi.org/10.1038/2101070a0 PMID:5950290

3. Contribution to Wuhan with SARS-CoV-2 IgG/IgM Assays. News release. YHLO, March 4, 2020. http://www.szyhlo.com/en/news_detail.php?menuid=75&id=125&from=singlemessage&isappinstalled=0

Research Paper

Clinical course and risk factors for recurrence of positive SARS-CoV-2 RNA: a retrospective cohort study from Wuhan, China

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ABSTRACT

The coronavirus disease 2019 (COVID-19) pandemic is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The objective of this study was to determine the clinical course and risk factors for patients showing recurrent SARS-CoV-2 RNA positivity. A total of 1087 COVID-19 patients confirmed by RT-PCR from February 24, 2020 to March 31, 2020 were retrospectively enrolled. Advanced age was significantly associated with mortality. In addition, 81 (7.6%) of the discharged patients tested positive for SARS-CoV-2 RNA during the isolation period. For patients with recurrent RT-PCR positivity, the median duration from illness onset to recurrence was 50 days. Multivariate regression analysis identified elevated serum IL-6, increased lymphocyte counts and CT imaging features of lung consolidation during hospitalization as the independent risk factors of recurrence. We hypothesized that the balance between immune response and virus toxicity may be the underlying mechanism of this phenomenon. For patients with a high risk of recurrence, a prolonged observation and additional preventative measures should be implemented for at least 50 days after illness onset to prevent future outbreaks.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1, 2]. As of April 18, 2020, 2,121,675 confirmed cases of COVID-19 and 142,299 related deaths have been reported from 213 countries according to the World Health Organization (WHO) [3]. Although several studies have summarized the epidemiological and clinical features of SARS-CoV-2 infection [4–6], and research is going on viral pathogenicity and mechanism. However, the exact origin of SARS-CoV-2 is controversial and a potential threat to a new outbreak [7, 8]. Furthermore, little is known regarding the immune response against SARS-CoV-2 infection, which in turn makes it difficult to assess complete recovery with no further risk of infection. The latter is a crucial factor in "flattening the curve" of COVID-19 and preventing additional outbreaks.

In the early stages of the COVID-19 outbreak that was located to Wuhan, China, the severe shortage and limitations in the detection and accuracy of the RT-PCR test restricted identification of infected patients. The diagnostic techniques have improved substantially since [9], and two or more multipoint throat-swabs are taken over 24 hours apart prior to discharge in order to minimize the false negative rate of RT-PCR tests [10]. Lan L et al. [11] reported that four medical professionals with COVID-19 who met the criteria for hospital discharge (including two consecutive negative RT-PCR results) reverted to SARS-CoV-2 positivity, indicating a potential asymptomatic carrier state. It remains to be determined whether patients with recurrent SARS-CoV-2 RNA positivity remain infectious after discharge. Furthermore, the clinical and radiological characteristics of the COVID-19 patients with recurrence is largely unknown.

Herein, we retrospectively analyzed 1087 patients with confirmed COVID-19 and explored the clinical course and risk factors of SARS-CoV-2 RNA recurrence by RT-PCR during post-discharge isolation.

RESULTS

Clinico-demographic characteristics of patients

A total of 1087 consecutive COVID-19 pneumonia patients positive for SARS-CoV-2 RNA were enrolled in this study. The median age of the cohort was 60 years (9 to 100 years; IQR - 49-69 years) and 635 (58.4%) of the patients were women. The majority (83.1%) of the cases were mild, whereas the proportion of severe and critical cases were 13.2% and 3.7% respectively. Most patients (874, 80.4%) had bilateral pulmonary infiltration on the chest CT, while 730 (67.2%) and 525 patients (48.3%) respectively showed ground-glass appearance and consolidation. In addition, 887 out of 1007 (88.1%) patients were positive for serum IgG, while 797 out of 1057 (75.4%) patients were positive for serum IgM against COVID 19.

The median length of hospitalization was 12 days (1-38 days; IQR, 8-17 days), and 20 patients died during hospitalization whereas 1067 were discharged. The total mortality rate was 1.8% and the discharge rate was 98.2%. Among the fatalities, 5 patients were graded as severe with mortality rate of 3.5%, and 15 were critical cases with a high mortality rate of 37.5%. The total mortality rate of the severe and critical cases was 10.6%. The median age of the deceased patients was 83 years (65 to 92 years; IQR, 79.3-87.8 years), which was significantly higher than that of the discharged patients ($P<0.001$). The main causes of deaths were multiple organ failure (MOSF), most commonly affecting the lungs, heart, liver and kidneys. Other clinical features, laboratory examinations and imaging findings are summarized in Supplementary Table 1.

Characteristics of patients with SARS-CoV-2 RNA recurrence

Eighty-one (7.6%) of the discharged patients reverted to SARS-CoV-2 RNA positive after two negative RT-PCR tests during the post-discharge isolation period. The median age of the recurring cases was 62 years (range 16-90 years; IQR, 50.5-68 years), and 51 (63.0%) were female. Twenty (24.7%) patients had accompanying hypertension and 9 (11.1%) had diabetes. Furthermore, 84.0% (68), 14.8% (12) and 1.2% (1) of the cases were mild, severe and critical respectively. Most of these patients had the initial symptoms of COVID-19 infection prior to positive SARS-CoV-2 RNA diagnosis, and only 15 (18.5%) were asymptomatic when first diagnosed. Before hospitalization, pulmonary infection was confirmed in 70 patients via CT scan, and 65 (65.3%) received anti-viral agents.

Laboratory and CT imaging results from the inpatient hospital-stay are summarized in Table 1. Seven (8.6%) patients had lymphocytopenia and only 4 (4.9%) patients had neutrophilia. High-sensitivity CRP was elevated in 8 (9.9%) patients, and the ESR, procalcitonin and IL-6 levels were increased in 27 (33.3%), 14 (17.3%) and 11 (13.6%) patients. Furthermore, 10 (12.3%) patients developed liver injury with elevated ALT, 4 (4.9%) demonstrated myocardial damage with elevated Accu-Tell troponin, and 11 (13.6%) patients had kidney injury with elevated serum BUN and creatinine levels. CT images revealed consolidation, ground-glass opacity and bilateral pulmonary infiltration in 49 (60.5%), 56 (69.1%) and 70 (86.4%) patients, respectively. Finally, 72 of 77 (93.5%) patients were positive for serum IgG, whereas 68 of 79 (86.1%) were positive for serum IgM against COVID-19.

Clinical course of patients with SARS-CoV-2 recurrence

As shown in Figure 1, the median length of hospitalization for patients that reverted to SARS-CoV-2 RNA positive state was 12 days (range, 4-27 days; IQR, 7-17 days). The median duration from discharge to recurrence was 9 days (range, 3-18 days; IQR, 7-10 days), and that from the onset of illness to RT-PCR confirmation was 11 days (range, 0-57 days; IQR, 1.5-21 days) (Figure 2A). In addition, the time from illness onset to complete RNA negative status was 33 days (range, 6-82 days; IQR, 20-41 days), and from illness onset to recurrence was 50 days (range, 21-95 days; IQR, 36.5-59.5 days). As shown in Figure 2B, the

Table 1. Clinico-demographic characteristics of patients with recurrence of SARS-CoV-2 RNA positivity.

Variables	No. (n=81)	Percentage (%)
General features		
Clinical severity of disease		
Mild	68	84.0%
Severe	12	14.8%
Critical	1	1.2%
Age		
Median (IQR)	62.0 (50.5-68.0)	
Gender		
Male	30	37.0%
Female	51	63.0%
Hypertension		
Yes	20	24.7%
No	61	75.3%
Diabetes		
Yes	9	11.1%
No	72	88.8%
Illness onset		
Fever		
Yes	41	50.6%
No	40	49.4%
Cough		
Yes	44	54.3%
No	37	45.7%
Chest congestion		
Yes	18	22.2%
No	63	77.8%
Weak		
Yes	34	42.5%
No	46	57.5%
Muscular soreness		
Yes	15	18.5%
No	66	81.5%
Pulmonary infection (CT)		
Yes	70	86.4%
No	5	6.2%
Unknown	6	7.4%
Anti-virus therapy		
Yes	53	65.4%
No	15	18.5%
Unknown	13	16.0%
In hospital		
Fever		
Yes(>=37.3 °C once or more)	18	22.2%
No	63	77.8%
Internal visceral dysfunctions		
Yes	30	37.0%
No	51	63.0%
Comorbid diseases		
Yes	46	56.8%
No	35	43.2%
White blood cell count, $\times 10^9$ per L		
<4		
4-10	6	7.4%
>10	71	87.7%
Unknown	3	3.7%
	1	1.2%
Neutrophil count, $\times 10^9$ per L		
<1.8	3	3.7%
1.8-6.3	73	90.1%
>6.3	4	4.9%
Unknown	1	1.2%
Lymphocyte count, $\times 10^9$ per L		
<1.1	7	8.6%

1.1-3.2	72	88.9%
>1.1	1	1.2%
Unknown	1	1.2%
Platelet count, $\times 10^9$ per L		
<125	4	4.9%
125-350	69	85.2%
>350	7	8.6%
Unknown	1	1.2%
ALT		
<40	71	87.7%
>=40	10	12.3%
Albumin		
<35	10	12.3%
>=35	71	87.7%
C-reactive protein		
<10	71	87.7%
>=10	8	9.9%
Unknown	2	2.5%
ESR 30min		
<20	13	16.0%
>=20	27	33.3%
Unknown	41	50.6%
Procalcitonin		
<=0.05	47	58.0%
>0.05	14	17.3%
Unknown	20	24.7%
D-dimer		
<0.5	47	58.0%
>=0.5	18	22.2%
Unknown	16	19.8%
BUN		
<=6.5	68	84.0%
>6.5	11	13.6%
Unknown	2	2.5%
Creatinine		
<90	68	84.0%
>=90	11	13.6%
Unknown	2	2.5%
Accu-Tell Troponin		
<15.6	47	58.0%
>=15.6	4	4.9%
Unknown	30	37.0%
IL-6		
<10	43	53.1%
>=10	11	13.6%
Unknown	27	33.3%
IgG		
Positive	72	88.9%
Negative	5	6.2%
Unknown	4	4.9%
IgM		
Positive	68	84.0%
Negative	11	13.6%
Unknown	2	2.5%
Imaging features		
Consolidation		
Yes	49	60.5%
No	32	39.5%
Ground-glass opacity		
Yes	56	69.1%
No	25	30.9%
Bilateral pulmonary infiltration		
Yes	70	86.4%
No	11	13.6%

median duration from initial RT-PCR diagnosis to recurrence was 36 days (range, 16-64 days; IQR, 26.5-45 days). In addition, the median duration between the initial diagnostic RT-PCR and complete RNA negative status was 17 days (range, 1-45 days; IQR, 8-29 days), while that between complete RNA negative status and recurrence was 12 days (range, 4-27 days; IQR, 7-17 days).

Amongst these 81 patients, 37 (45.7%) received oxygen support. However, no invasive mechanical ventilation (IMV) or IMV with extracorporeal membrane

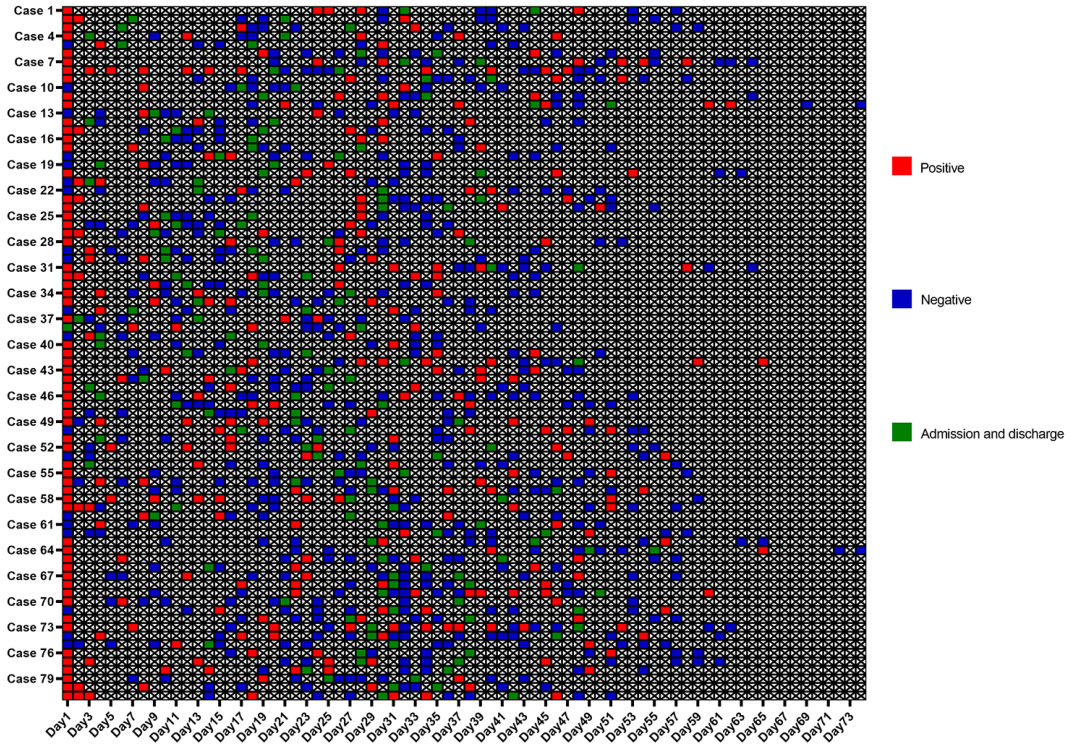

Figure 1. Individual duration of viral shedding and positive SARS-CoV-2 RNA recurrence from illness onset after discharge. The timing and results of RT-PCR examinations for SARS-CoV-2 RNA in details. SARS-CoV-2=severe acute respiratory syndrome coronavirus 2. RT-PCR=reverse transcription-polymerase chain reaction.

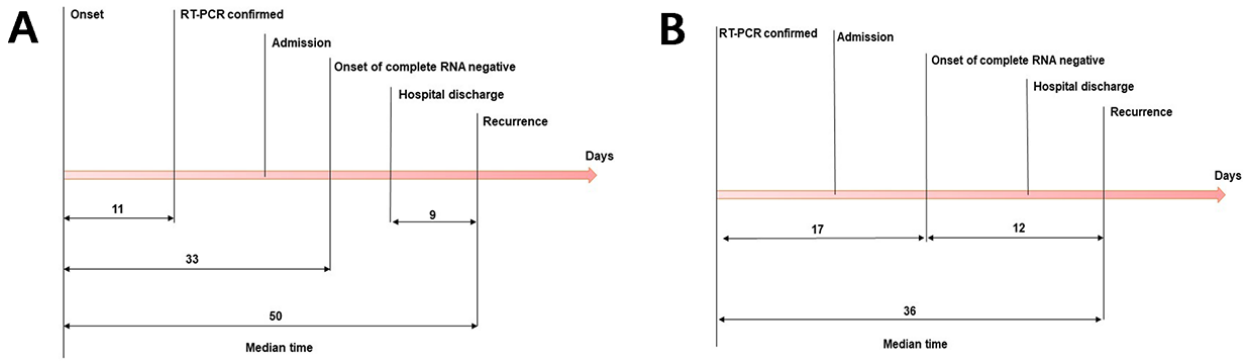

Figure 2. The median duration of different stages in patients with recurrence of positive SARS-CoV-2 RNA after discharge. (**A**) The median duration from illness onset to initial RT-PCR confirmation, onset of complete RNA negative status and recurrent RT-PCR positivity after discharge, and from discharge to recurrence. (**B**) The median duration from initial RT-PCR confirmation to onset of complete RNA negative status and recurrent RT-PCR positivity after discharge, and from onset of complete RNA negative status to recurrence. SARS-CoV-2=severe acute respiratory syndrome coronavirus 2. RT-PCR=reverse transcription-polymerase chain reaction.

oxygenation (ECMO) was used. The optimal antiviral therapy was administered in 69 (85.2%) patients, including arbidol hydrochloride (40 patients, 49.4%), interferon alfa (17 patients, 21.0%), entecavir/tenofovir (7 patients, 8.6%) and oseltamivir (5 patients, 6.2%). Fifty-one patients (63%) were treated with Chinese patented drugs, such as Lianhuaqingwen capsule. Vitamin C was given to 41 (50.6%) patients, and immunomodulators like thymopentin and immunoglobulin were administrated to 8 (9.9%) patients.

Associated risk factors with recurrence of positive SARS-CoV-2 RNA

As shown in Table 2, positive SARS-CoV-2 RNA recurrence correlated positively with serum IL-6 level (P=0.010) and CT imaging depicting consolidation (P=0.031). In the univariate analysis, elevated lymphocyte count (P=0.194, OR=1.644; 95% CI, 0.776-3.484), elevated serum IL-6 (P=0.013, OR=2.504; 95% CI, 1.218-5.150), consolidation on CT imaging (P=0.033, OR=1.655; 95% CI, 1.042-2.629) and bilateral pulmonary infiltration (P=0.196, OR=1.540; 95% CI, 0.800-2.966) were identified as potential risk factors for recurrence of SARS-CoV-2 RNA positivity (Table 3). Multivariate analysis concluded that elevated lymphocyte count (P=0.038, OR=2.321; 95% CI, 1.048-5.138), serum IL-6 level (P=0.004, OR=3.050; 95% CI, 1.432-6.499) and consolidation features on CT imaging (P=0.038, OR=1.641; 95% CI, 1.028-2.620) were the independent risk factors of recurrence (Table 4).

DISCUSSION

In this study, we have provided comprehensive data on the demographic and clinical characteristics of 1087 consecutive COVID-19 patients from Wuhan, China. The majority (83.1%) of the cases in our cohort were mild, and the overall mortality rate of the severe and critical cases was 10.6%. The mortality rate of the entire cohort was 1.8%, which is consistent to one previous study [4] but lower than that reported in other studies [5, 12]. This difference can be partly attributed to the higher proportion of severe cases in the other cohorts, as well as the greater medical resources that were allocated in the later stages of this pandemic wherein we enrolled patients for our study. Liang WH et al. [13] reported that the mortality of COVID-19 patients outside of the Hubei Province was limited to 0.3%, as strict public health interventions were initiated in order to prevent further outbreak outside Hubei and adequate medical resources were provided for treatment. In agreement with previous studies that identified older age as a risk factor of mortality in COVID-19 patients [6, 14], the median age of the deceased patients in our cohort was 83 years, distinctly higher than that of the discharged patients (P<0.001), which further suggests that a higher age was significantly associated with mortality.

Among the 1067 patients that were discharged on the basis of negative SARS-CoV-2 RNA results, 81 (7.6%) patients reverted to positive state during their isolation period. Similar findings have been reported previously [11, 15, 16]. However, Yuan J et al. [16] reported a higher repeat positivity rate of 14.5% after discharge, which could be on account the smaller cohort of enrolled patients. These persistent asymptomatic viral carriers may pose a risk for potential future outbreaks despite unprecedented public health interventions [17]. Therefore, we explored the clinical course and risk predictors for recurrent SARS-CoV-2 PCR positivity in order to provide new insights into the disease and help guide the clinical practice against future outbreaks.

In our study, the median duration of viral shedding for patients with positive SARS-CoV-2 RNA recurrence was 33 days from the onset of illness to complete RNA negative status. However, the median duration from illness onset to SARS-CoV-2 RNA reversion was 50 days. Previous studies have reported on duration of viral shedding. Zhou F et al. [6] reported a 20 day median duration of viral shedding in survivors and the longest observed duration was 37 days. Furthermore, Zhou B et al. [18] reported that the median duration of viral shedding was 31 days from illness onset in severe COVID-19 patients. Xu K et al. [19] further showed that 3 out of 4 COVID-19 patients had viral RNA clearance within 21 days of illness onset, and male gender, older age, hypertension, delayed hospital admission, severe illness upon admission, invasive mechanical ventilation and corticosteroid treatment were risk factors for prolonged viral RNA clearance. Our findings underscore the importance of a prolonged treatment or isolation for patients at increased risk of recurrent SARS-CoV-2 RNA positivity.

Nevertheless, we found that age and comorbidities that were previously described to be risk factors of mortality [14] were not identified as significant risk factors when compared to patients without reversion. Instead, high serum IL-6 levels, lymphocyte count greater than $1.1*10^8$ /L and consolidation on CT imaging during hospitalization were associated with a higher likelihood of recurrent SARS-CoV-2 RNA positivity after discharge. This is consistent with a previous study that showed that the lymphocyte count prior to discharge was positively correlated with the time to virus reappearance, which confirms the role of lymphocytes in the potential recurrence of SARS-CoV-2 RNA positivity [16]. Other factors that influence the host defense against viral infections, such as clinical severity

Table 2. Correlations between clinical characteristics and recurrence of SARS-CoV-2 RNA positivity in discharged patients.

Variables	No. (n=1067)	No recurrence n=986	Recurrence n=81	P value
General features				
Clinical severity of disease				
Mild	903	835	68	0.684
Severe	139	127	12	
Critical	25	24	1	
Age				0.700
Median (IQR)	60.0 (49.0-68.0)	60.0 (49.0-68.0)	62.0 (50.5-68.0)	
Gender				
Male	440	410	30	0.424
Female	627	576	51	
Hypertension				
Yes	331	311	20	0.200
No	736	675	61	
Diabetes				
Yes	135	126	9	0.664
No	932	860	72	
In hospital				
Fever				
Yes(>=37.3°C once or more)	246	228	18	0.853
No	821	758	63	
Internal visceral dysfunctions				
Yes	343	313	30	0.327
No	724	673	51	
Comorbid diseases				
Yes	560	514	46	0.419
No	507	472	35	
White blood cell count, $\times 10^9$ per L				
<4	100	94	6	0.579
4-10	927	856	71	
>10	24	21	3	
Unknown	16	15	1	
Neutrophil count, $\times 10^9$ per L				
<=6.3	994	918	76	1.000
>6.3	57	53	4	
Unknown	16	15	1	
Lymphocyte count, $\times 10^9$ per L				
<=1.1	158	150	8	0.190
>1.1	894	821	72	
Unknown	15	15	1	
Platelet count, $\times 10^9$ per L				
<125	44	40	4	
125-350	956	886	69	0.297
>350	52	45	7	
Unknown	15	15	1	
ALT				
<40	852	781	71	0.202
>=40	181	171	10	
Unknown	34	34	0	
Albumin				
<35	154	144	10	0.502
>=35	880	809	71	
Unknown	33	33	0	
C-reactive protein				
<10	914	843	71	0.653
>=10	121	113	8	
Unknown	32	30	2	
ESR 30min				
<20	176	163	13	0.420
>=20	282	255	27	
Unknown	609	568	41	
Procalcitonin				0.571

<=0.05	589	542	47	
>0.05	207	193	14	
Unknown	271	251	20	
D-dimer				
<0.5	507	460	47	0.339
>=0.5	250	232	18	
Unknown	310	294	16	
BUN				
<=6.5	853	785	68	0.822
>6.5	148	137	11	
Unknown	66	64	2	
Creatinine				
<90	907	839	68	0.150
>=90	94	83	11	
Unknown	66	64	2	
Accu-Tell Troponin				
<15.6	590	543	47	1.000
>=15.6	54	50	4	
Unknown	423	393	30	
IL-6				
<10	552	509	43	0.010
>=10	63	52	11	
Unknown	452	425	27	
Imaging features				
Consolidation				
Yes	520	471	49	0.031
No	541	509	32	
Unknown	6	6	0	
Ground-glass opacity				
Yes	720	664	56	0.798
No	341	316	25	
Unknown	6	6	0	
Bilateral pulmonary infiltration				
Yes	859	789	70	0.193
No	202	191	11	
Unknown	6	6	0	

of the disease, CRP, D-dimer level etc., were not significantly different between the recurrent versus non-recurrent groups. IL-6 is one of the major pro-inflammatory cytokines that are instrumental in clearing pathogens. However, the rapid multiplication of SARS-CoV-2 in the lower respiratory tract leads to excessive IL-6 production, which triggers an acute severe systemic inflammatory response known as cytokine release syndrome (CRS) [20]. In fact, the increased serum IL-6 levels in severe and critical COVID-19 patients is associated with poor outcomes [21, 22], which was also observed during severe acute respiratory syndrome (SARS) outbreak [23]. Concurrently, lymphopenia is also common in patients with COVID-19, especially in severe and critical cases [5, 22, 24], suggesting a dysregulated immune response in this sub-cohort. In our study however, only 175 (16.1%) patients showed a decrease in lymphocyte count, which again may be can be attributed to the fewer severe cases. Interestingly, the discharged patients with recurrence of positive SARS-CoV-2 RNA had an elevated serum IL-6 level and lymphocyte count compared to those with no recurrence, indicating that the immune system may still be actively involved in clearing the infection. It is also possible that the immune responses can suppress but not completely eradicate SARS-CoV-2, which may have led to the false-negative results due to lower viral loads. Once the virus started replicating again, the RT-PCR results reverted to positive in the discharged patients.

The chest CT imaging of COVID-19 pneumonia is a useful preliminary diagnostic tool that has lowered the rate of missed diagnoses [25]. Features of consolidation on CT imaging are associated with critical disease [26]. Progression of consolidation might indicate further infiltration of the lung parenchyma and lung interstitium due to virus invasion into the respiratory epithelium, which is characterized by diffuse alveolar damage and necrotizing bronchitis. This eventually leads to complete permeation of the alveoli with the inflammatory exudate [27, 28]. Therefore, SARS-CoV-2 may persist in the respiratory epithelium during lung consolidation in the recovery phase of the infection, which eventually results in the recurrence of positive SARS-CoV-2 RNA after discharge. Interestingly, most patients with recurrence had fluctuating positive and

Table 3. Univariate regression analysis for risk factors of patients with recurrence of SARS-CoV-2 RNA positivity.

Variables	No.	Univariate OR (95% CI)	P value
Age	1067	1.000(0.985-1.015)	0.995
Gender	1067	1.210(0.757-1.933)	0.425
Clinical severity of disease	1067	0.976(0.579-1.645)	0.927
Hypertension	1067	0.712(0.422-1.200)	0.202
Diabetes	1067	0.853(0.416-1.749)	0.665
Fever	1067	0.950(0.551-1.637)	0.853
Internal visceral dysfunctions	1067	1.265(0.790-2.025)	0.328
Comorbid diseases	1067	1.207(0.764-1.906)	0.420
Neutrophil count, $\times 10^9$ per L	1051	0.912(0.321-2.587)	0.862
Lymphocyte count, $\times 10^9$ per L	1051	1.644(0.776-3.484)	**0.194**
Platelet count, $\times 10^9$ per L	1051	1.417(0.676-2.969)	0.356
ALT	1033	0.643(0.325-1.273)	0.205
Albumin	1034	1.264(0.637-2.508)	0.503
C-reactive protein	1035	0.841(0.394-1.792)	0.653
ESR 30min	458	1.328(0.666-2.647)	0.421
Procalcitonin	796	0.837(0.450-1.553)	0.572
D-dimer	757	0.759(0.431-1.337)	0.340
Accu-Tell Troponin	644	0.924(0.320-2.671)	0.884
IL-6	615	2.504(1.218-5.150)	0.013
Consolidation	1061	1.655(1.042-2.629)	0.033
Ground-glass opacity	1061	1.066(0.653-1.740)	0.798
Bilateral pulmonary infiltration	1061	1.540(0.800-2.966)	**0.196**

Table 4. Multivariate regression analysis for risk factors of patients with recurrence of SARS-CoV-2 RNA positivity.

Variables	Multivariate OR (95% CI)	P value
IL-6		
<10	Reference	
>=10	3.050(1.432-6.499)	0.004
Consolidation		
No	Reference	
Yes	1.641(1.028-2.620)	0.038
Lymphocyte count, $\times 10^9$ per L		
<=1.1	Reference	
>1.1	2.321(1.048-5.138)	0.038
Bilateral pulmonary infiltration		
No	Reference	
Yes	1.482(0.764-2.871)	0.244

negative results in the course of the disease, especially in cases 7, 8 and 41 (Figure 1). This is a potential sign of recurrent SARS-CoV-2 positivity after discharge, and also partly ruled out the randomly error probability in RT-PCR detection for one case. Thus, the infected patients may have already been immune to the virus and require a period for complete recovery. However, if the immune response cannot deal with the recurrence, further treatment may be still needed.

Limitations

This study has a few limitations that ought to be noted. First, this study was conducted at a single-center hospital which may have introduced a selection bias that influenced the clinical outcomes. A larger multi-center or even global cohort study of COVID-19 patients would help further define the clinical characteristics and risk factors of recurrence. Second, only multipoint throat-swab

specimens were tested which increases the risk of false negative results. Therefore, multisite samples should be collected for RT-PCR detection, such as the fecal SARS-CoV-2 RNA test for patients with gastrointestinal symptoms [29]. Third, the retrospective design and initial lack of guidelines for drug administration made it difficult to analyze the impact of treatment regimens on the recurrence of positive SARS-CoV-2 RNA.

CONCLUSIONS

Elevated lymphocyte counts and serum IL-6 level, and consolidation on chest CT were associated with a greater risk of recurrent SARS-CoV-2 RNA positivity, possibly due to a balance between immune regulation and virus toxicity. For patients with a higher risk of recurrence, a prolonged treatment or isolation period for at least 50 days after illness onset is recommended in order to identify patients that may pose a risk for future outbreaks.

MATERIALS AND METHODS

Study design and participants

A total of 1087 consecutive COVID-19 patients diagnosed by SARS-CoV-2 RNA detection in accordance with the interim guidelines of World Health Organization at the Guanggu Branch of Hubei Province Maternity and Childcare Hospital (Wuhan, China) were retrospectively enrolled. All patients had been discharged or had died between February 24, 2020 and March 31, 2020. This study was approved by the Research Ethics Committee of Guanggu Branch of Hubei Province Maternity and Childcare Hospital and was granted with a waiver of informed consent from study participants.

Data collection and follow-up

The epidemiological, radiographic, laboratory, treatment and treatment outcome data of these patients were extracted from medical records and through direct communication in order to establish a database. The SARS-CoV-2 RNA RT-PCR records from discharge to April 15, 2020 were obtained from the Health Wuhan App, a database containing all real-time results about SARS-CoV-2 RNA tests conducted in Wuhan. The patients were assigned a number for confidentiality. All data were evaluated by two authors (JC and QC) and thereafter by a third researcher (NP) in case of any differences in interpretation.

Clinical tests

In accordance with the standard procedure, throat-swab specimens were obtained and tested for SARS-CoV-2 infection using RT-PCR by the Academy of Military Medical Sciences and hospital laboratory [14]. The test was repeated during the hospital stay and after clinical remission of symptoms at 24-hour intervals. In addition, serum levels of SARS-CoV-2-specific IgM/IgG measured during hospitalization with the indirect enzyme-linked immunosorbent assay (ELISA) protocol using the N protein of SARS-CoV-2 as the coating antigen. Routine blood tests were performed to determine complete blood counts (including white blood cells, neutrophils, lymphocytes, monocytes and platelets), biochemical indices (liver function, renal function and electrolyte levels), coagulation indices, high-sensitivity C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), procalcitonin, myocardial enzymes, D-dimer and interleukin-6 (IL-6). Computed tomography (CT) scans were routinely performed as recommended by the attending physician.

Clinical definitions

The patients were discharged based on the following criteria: 1) no fever for at least three days, 2) remission of respiratory symptoms, 3) amelioration of pulmonary inflammation on the chest CT scan, 4) two negative SARS-CoV-2 RNA tests at least 24 hours apart, 5) overall good constitution.

The severity of COVID-19 was defined according to the Chinese management guidelines for COVID-19 (version 6.0) [10]. Fever was defined as axillary temperature of at least 37.3°C. Comorbidities during hospitalization included hypertension, diabetes, hypoproteinaemia (< 25 g/L), coagulopathy (3-second increase in prothrombin time or a 5-second increase of activated partial thromboplastin time), hyperuricemia (blood trioxypurine > 420 μmol/L or > 360 μmol/L in males and females respectively), anemia (according to WHO guidelines [30]), acute respiratory distress syndrome (ARDS; diagnosed according to the Berlin Definition [31]), acute liver failure (diagnosed according to EASL Clinical Practical Guidelines [32]), acute kidney injury (diagnosed according to the KDIGO clinical practice guidelines [33]), and acute cardiac injury (diagnosed as previously reported [6])

Statistical analysis

Continuous and categorical variables were respectively presented as median with interquartile range (IQR) and counts with percentages. The differences between the recurrence and non-recurrence groups were compared using the Pearson Chis-squared test, Fisher's exact test or Mann-Whitney U test as appropriate. The risk factors associated with the recurrence of positive SARS-CoV-2 RNA were identified using univariate analysis, and

variables with $P < 0.2$ were selected for multivariate logistic regression model. Missing data was not included in any of the analyses. A two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the SPSS v21.0 software (IBM Corporation, Armonk, NY, USA), and the figures were plotted using the GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA).

AUTHOR CONTRIBUTIONS

JC, XX, NP, and ZC conceived and designed the research. JC, XX, JH, FX, HL, NL, HZ, JL, JX, NP and ZC collected and analyzed data. JC, QC, NP, and ZC participated in evaluation of results. JC, QC, and NP wrote the manuscript, and all authors contributed to manuscript revision, read and approved the submitted version.

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CONFLICTS OF INTEREST

We declare no conflicts of interest.

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REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

2. Phelan AL, Katz R, Gostin LO. The novel coronavirus originating in Wuhan, China: challenges for global health governance. JAMA. 2020. [Epub ahead of print]. https://doi.org/10.1001/jama.2020.1097 PMID:31999307

3. World Health Organization. Novel Coronavirus (2019-nCoV) https://www.who.int/emergencies/diseases/novel-coronavirus-2019.

4. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

5. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

6. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

7. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. Nat Med. 2020; 26:450–52. https://doi.org/10.1038/s41591-020-0820-9 PMID:32284615

8. Forster P, Forster L, Renfrew C, Forster M. Phylogenetic network analysis of SARS-CoV-2 genomes. Proc Natl Acad Sci U S A. 2020; 117:9241–9243. https://doi.org/10.1073/pnas.2004999117 PMID:32269081

9. Li Y, Yao L, Li J, Chen L, Song Y, Cai Z, Yang C. Stability issues of RT-PCR testing of SARS-CoV-2 for hospitalized patients clinically diagnosed with COVID-19. J Med Virol. 2020; 92:903–08. https://doi.org/10.1002/jmv.25786 PMID:32219885

10. National Health Commission of the People's Republic of China. Chinese management guideline for COVID-19 (version 6.0). Feb 19, 2020. http://www.nhc.gov.cn/yzygj/s7653p/202002/8334a8326dd94d329df351d7da8aefc2/files/b218cfeb1bc54639af227f922bf6b817.pdf.

11. Lan L, Xu D, Ye G, Xia C, Wang S, Li Y, Xu H. Positive RT-PCR test results in patients recovered from COVID-19. JAMA. 2020; 323:1502–03. https://doi.org/10.1001/jama.2020.2783 PMID:32105304

12. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases

of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

13. Liang WH, Guan WJ, Li CC, Li YM, Liang HR, Zhao Y, Liu XQ, Sang L, Chen RC, Tang CL, Wang T, Wang W, He QH, et al. Clinical characteristics and outcomes of hospitalised patients with COVID-19 treated in hubei (epicentre) and outside hubei (non-epicentre): a nationwide analysis of China. Eur Respir J. 2020; 55:2000562. https://doi.org/10.1183/13993003.00562-2020 PMID:32269086

14. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Zhou X, Du C, Zhang Y, Song J, Wang S, et al. Risk factors associated with acute respiratory distress syndrome and death in patients with coronavirus disease 2019 pneumonia in Wuhan, China. JAMA Intern Med. 2020; 180:1–11. https://doi.org/10.1001/jamainternmed.2020.0994 PMID:32167524

15. Chen D, Xu W, Lei Z, Huang Z, Liu J, Gao Z, Peng L. Recurrence of positive SARS-CoV-2 RNA in COVID-19: a case report. Int J Infect Dis. 2020; 93:297–99. https://doi.org/10.1016/j.ijid.2020.03.003 PMID:32147538

16. Yuan J, Kou S, Liang Y, Zeng J, Pan Y, Liu L. PCR assays turned positive in 25 discharged COVID-19 patients. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa398 PMID:32266381

17. Pan A, Liu L, Wang C, Guo H, Hao X, Wang Q, Huang J, He N, Yu H, Lin X, Wei S, Wu T. Association of public health interventions with the epidemiology of the COVID-19 outbreak in Wuhan, China. JAMA. 2020; 323:1–9. https://doi.org/10.1001/jama.2020.6130 PMID:32275295

18. Zhou B, She J, Wang Y, Ma X. The duration of viral shedding of discharged patients with severe COVID-19. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa451 PMID:32302000

19. Xu K, Chen Y, Yuan J, Yi P, Ding C, Wu W, Li Y, Ni Q, Zou R, Li X, Xu M, Zhang Y, Zhao H, et al. Factors associated with prolonged viral RNA shedding in patients with COVID-19. Clin Infect Dis. 2020. 71:799-806. https://doi.org/10.1093/cid/ciaa351 PMID:32271376

20. Liu B, Li M, Zhou Z, Guan X, Xiang Y. Can we use interleukin-6 (IL-6) blockade for coronavirus disease 2019 (COVID-19)-induced cytokine release syndrome (CRS)? J Autoimmun. 2020; 111:102452. https://doi.org/10.1016/j.jaut.2020.102452 PMID:32291137

21. Chen X, Zhao B, Qu Y, Chen Y, Xiong J, Feng Y, Men D, Huang Q, Liu Y, Yang B, Ding J, Li F. Detectable serum SARS-CoV-2 viral load (RNAaemia) is closely correlated with drastically elevated interleukin 6 (IL-6) level in critically ill COVID-19 patients. Clin Infect Dis. 2020. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa449 PMID:32301997

22. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang X, Zhang M, Wu S, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest. 2020; 130:2620–29. https://doi.org/10.1172/JCI137244 PMID:32217835

23. Zhang Y, Li J, Zhan Y, Wu L, Yu X, Zhang W, Ye L, Xu S, Sun R, Wang Y, Lou J. Analysis of serum cytokines in patients with severe acute respiratory syndrome. Infect Immun. 2004; 72:4410–15. https://doi.org/10.1128/IAI.72.8.4410-4415.2004 PMID:15271897

24. Yang P, Ding Y, Xu Z, Pu R, Li P, Yan J, Liu J, Meng F, Huang L, Shi L, Jiang T, Qin E, Zhao M, et al. Epidemiological and clinical features of COVID-19 patients with and without pneumonia in Beijing, China. medRxiv. 2020. https://doi.org/10.1101/2020.02.28.20028068

25. Li Y, Xia L. Coronavirus disease 2019 (COVID-19): role of chest CT in diagnosis and management. AJR Am J Roentgenol. 2020; 214:1280–86. https://doi.org/10.2214/AJR.20.22954 PMID:32130038

26. Lyu P, Liu X, Zhang R, Shi L, Gao J. The performance of chest CT in evaluating the clinical severity of COVID-19 pneumonia: identifying critical cases based on CT characteristics. Invest Radiol. 2020; 55:412–21. https://doi.org/10.1097/RLI.0000000000000689 PMID:32304402

27. Zhou S, Wang Y, Zhu T, Xia L. CT features of coronavirus disease 2019 (COVID-19) pneumonia in 62 patients in Wuhan, China. AJR Am J Roentgenol. 2020; 214:1287–94. https://doi.org/10.2214/AJR.20.22975 PMID:32134681

28. Koo HJ, Lim S, Choe J, Choi SH, Sung H, Do KH. Radiographic and CT features of viral pneumonia. Radiographics. 2018; 38:719–39. https://doi.org/10.1148/rg.2018170048 PMID:29757717

29. Cheung KS, Hung IF, Chan PP, Lung KC, Tso E, Liu R, Ng YY, Chu MY, Chung TW, Tam AR, Yip CC, Leung KH, Fung AY, et al. Gastrointestinal manifestations of SARS-CoV-2 infection and virus load in fecal samples from a Hong Kong cohort: systematic review and

meta-analysis. Gastroenterology. 2020; 159:81–95. https://doi.org/10.1053/j.gastro.2020.03.065 PMID:32251668

30. WHO. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity. Vitamin and Mineral Nutrition Information System. Geneva, World Health Organization, 2011. (WHO/NMH/NHD/MNM/11.1). http://www.who.int/vmnis/indicators/haemoglobin.pdf

31. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS, and ARDS Definition Task Force. Acute respiratory distress syndrome: the berlin definition. JAMA. 2012; 307:2526–33. https://doi.org/10.1001/jama.2012.5669 PMID:22797452

32. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu; Clinical practice guidelines panel, Wendon, J; Panel members, Cordoba J, Dhawan A, Larsen FS, Manns M, Samuel D, Simpson KJ, Yaron I; EASL Governing Board representative, Bernardi M. EASL Clinical Practical Guidelines on the management of acute (fulminant) liver failure. J Hepatol. 2017; 66:1047–1081. https://doi.org/10.1016/j.jhep.2016.12.003 PMID:28417882

33. Khwaja A. KDIGO clinical practice guidelines for acute kidney injury. Nephron Clin Pract. 2012; 120:c179–84. https://doi.org/10.1159/000339789 PMID:22890468

SUPPLEMENTARY MATERIAL

Supplementary Table

Supplementary Table 1. Clinico-demographic characteristics of all patients with COVID-19 confirmed by RT-PCR.

Variables	No. (n=1087)	Percentage (%)
General features		
Clinical severity of disease		
Mild	903	83.1%
Severe	144	13.2%
Critical	40	3.7%
Age		
Median (IQR)	60.0 (49.0-69.0)	
Gender		
Male	452	41.6%
Female	635	58.4%
Hypertension		
Yes	337	31.0%
No	750	69.0%
Diabetes		
Yes	137	12.6%
No	950	87.4%
In hospital		
Fever		
Yes(>=37.3°C once or more)	254	23.4%
No	833	76.6%
Internal visceral dysfunctions		
Yes	363	33.4%
No	724	66.6%
Comorbid diseases		
Yes	580	53.4%
No	507	46.6%
White blood cell count, $\times 10^9$ per L		
<4	102	9.4%
4-10	938	86.3%
>10	31	2.9%
Unknown	16	1.5%
Neutrophil count, $\times 10^9$per L		
<=6.3	1005	92.5%
>6.3	66	6.1%
Unknown	16	1.5%
Lymphocyte count, $\times 10^9$per L		
<=1.1	175	16.1%
>1.1	896	82.4%
Unknown	16	1.5%
Platelet count, $\times 10^9$per L		
<125	52	4.8%
125-350	967	89.0%
>350	52	4.8%
Unknown	16	1.5%
ALT		
<40	867	79.8%
>=40	185	17.0%
Unknown	35	3.2%
Albumin		
<35	171	15.7%
>=35	883	81.2%
Unknown	33	3.0%
C-reactive protein		
<10	919	84.5%
>=10	136	12.5%
Unknown	32	2.9%
ESR 30min		
<20	178	16.4%

>=20	290	26.7%
Unknown	619	56.9%
Procalcitonin		
<=0.05	594	54.6%
>0.05	221	20.3%
Unknown	272	25.0%
D-Dimer		
<0.5	510	46.9%
>=0.5	265	24.4%
Unknown	312	28.7%
BUN		
<=6.5	859	79.0%
>6.5	162	14.9%
Unknown	66	6.1%
Creatinine		
<90	917	84.4%
>=90	104	9.6%
Unknown	66	6.1%
Accu-Tell Troponin		
<15.6	593	54.6%
>=15.6	66	6.1%
Unknown	428	39.4%
IL-6		
<10	555	51.1%
>=10	76	7.0%
Unknown	456	42.0%
IgG		
Positive	887	81.6%
Negative	120	11.0%
Unknown	80	7.4%
IgM		
Positive	797	73.3%
Negative	260	23.9%
Unknown	30	2.8%
Imaging features		
Consolidation		
Yes	525	48.3%
No	551	50.7%
Unknown	11	1.0%
Ground-glass opacity		
Yes	730	67.2%
No	346	31.8%
Unknown	11	1.0%
Bilateral pulmonary infiltration		
Yes	874	80.4%
No	202	18.6%
Unknown	11	1.0%

Review

Neurological manifestations in COVID-19 and its possible mechanism

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ABSTRACT

In December 2019, the first cases of the acute respiratory illness now known as Corona Virus Disease 2019 (COVID-19) occurred in Wuhan, Hubei Province, China. The main clinical manifestations of COVID-19 are a fever, dry cough and general weakness, although in some patients, a headache, tight chest, diarrhea, etc. are the first clinical manifestations. Neurological practice is involved in all aspects of medicine, from primary care for patients with migraines to consultations with patients in the intensive care unit. Few disorders spare the nervous system, and newly emerging infections are no exception. As neurologists, we are concerned about the effects of SARS-CoV-2 infections on the nervous system. Multiple neuropathy, rhabdomyolysis, cerebrovascular disease, central nervous system infections and other common neurological diseases require attention during this outbreak.

INTRODUCTION

In December 2019, a number of unexplained cases of pneumonia occurred in Wuhan, China, and rapidly spread to other parts of China, then to Europe, North America, Asia and most of the world. This outbreak was confirmed to be caused by a novel coronavirus – severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1, 2]. On March 11, 2020, the World Health Organization declared Corona Virus Disease 2019 (COVID-19) as a pandemic [3]. As of May 7, 2020, there were 3,672,238 confirmed cases of COVID-19 and 254,045 deaths due to the disease globally [4]. The most common symptoms in patients diagnosed with SARS-CoV-2 infections are a fever and dry cough [5, 6]. Infections caused by SARS-CoV-2 exhibit many clinical similarities to those caused by SARS-CoV, such as a fever, dry cough and diarrhea during the prodromal phase [7–9].

In addition to the typical respiratory symptoms, some SARS patients have had neurological problems [10, 11]. Similarly, in some patients diagnosed with COVID-19, headaches, muscle aches, confusion and seizures have been the first clinical manifestations [12, 13]. In COVID-19 epidemic areas, people who have had close contact with diagnosed COVID-19 patients should be alert to the possibility of SARS-CoV-2 infection if they develop neurological symptoms such as headaches, slurred speech, hemiplegia and disturbances of consciousness. This article reviews the epidemiology of SARS-CoV-2 infections, the neurological diseases related to SARS-CoV-2, and the possible mechanisms behind these relationships.

SARS-CoV-2

SARS-CoV-2, originally named 2019-nCoV, belongs to the broader family of coronaviruses [1, 2]. Coronaviruses are enveloped, positive-stranded RNA viruses that belong to the family Coronaviridae and the order Nidovirales. Six coronavirus species are known to cause respiratory, enteric, hepatic and neurologic diseases. Four of these

viruses are prevalent – 229E, OC43, NL63 and HKU1 – and typically cause common cold symptoms in immuno-competent individuals [14]. The two other strains – SARS-CoV [15] and Middle East respiratory syndrome coronavirus (MERS-CoV) [16] – are zoonotic in origin, and have been linked to sometimes fatal illness. SARS-CoV-2 is the seventh member of the coronavirus family. Zhou et al. found that SARS-CoV-2 was 96% identical at the whole-genome level to a bat coronavirus, so SARS-CoV-2 may have originated in bats [17].

An epidemic or outbreak can occur when the agent (pathogen), population (hosts) and environment create an ideal situation for spread [18]. The current evidence suggests that SARS-CoV-2 may have spread to humans via wild animals sold illegally in the Huanan Seafood Wholesale Market [19]. The extent of human-to-human transmission of SARS-CoV-2 was unclear at first, but now there is evidence of human-to-human transmission [5, 20, 21]. The main sources of infection are SARS-CoV-2-infected patients, including those who are asymptomatic [22]. The routes of transmission include droplet transmission, contact transmission and aerosol transmission [23, 24]. In a recent study, SARS-CoV-2 was detected in stool samples from patients with abdominal symptoms [20, 25], so some scholars have proposed that SARS-CoV-2 could spread *via* fecal-oral transmission. Further environmental studies will be needed to determine whether the virus remains viable under conditions that would favor fecal-oral transmission [26]. SARS-CoV-2 has not been confirmed to be transmitted vertically from mother to child [27]. Based on the available data, a Chinese team estimated a basic reproduction number (R0) of 3.77 for SARS-CoV-2, basically confirming that the new coronavirus is more contagious than SARS [28].

SARS-CoV-2 employs a densely glycosylated, homotrimeric class I fusion spike (S) protein to enter host cells. The S protein exists in a metastable prefusion conformation that undergoes a dramatic structural re-arrangement to fuse the viral membrane with the host cell membrane [29, 30]. Epidemiological data indicate that the population is generally susceptible to SARS-CoV-2 [31]. Therefore, it is necessary for individuals to wash or disinfect their hands frequently, go outside less, wear a mask, avoid group activities, stay away from patients with COVID-19, maintain good living habits and keep an optimistic attitude [32–34].

Neurological disease

SARS-CoV-2 has been reported to be associated with Guillain-Barré syndrome, rhabdomyolysis, acute cerebrovascular disease, central nervous system infections and other neurological diseases. Four formal reports have described neurological problems in SARS patients, including polyneuropathy [35], myopathy and rhabdomyolysis [36], large artery ischemic stroke [37] and central nervous system infections [38]. Human coronaviruses (HCoVs) can naturally reach the central nervous system, and could potentially cause neurological symptoms. Among the coronavirus-induced animal diseases, feline infectious peritonitis virus, mouse hepatitis virus and hemagglutinating encephalomyelitis virus can all reach the central nervous system and induce different types of neuropathologies [10, 11]. The structure of SARS-CoV-2 is similar to that of the SARS virus, and both viruses invade the human body through the angiotensin converting enzyme II (ACE2) receptor. Thus, in this paper, we mainly describe the neurological diseases associated with SARS-CoV-2, but also briefly introduce the neurological diseases associated with SARS.

Neuromuscular manifestations

Polyneuropathy

Polyneuropathy, also known as peripheral neuropathy, is multiple-nerve damage of the extremities. The clinical manifestations are mostly distal symmetrical motor sensory dysfunction and autonomic nerve dysfunction [39]. The causes of polyneuropathic disorders include metabolic, toxic, infectious, inflammatory, autoimmune and genetic conditions [40]. Zhao et al. reported a case of COVID-19 initially presenting with acute Guillain-Barré syndrome (GBS). The female patient aged 61 years presented with acute weakness in both legs and severe fatigue. She received intravenous immuno-globulin, antiviral drugs of arbidol, lopinavir, and ritonavir, and supportive care. After 30 days of treatment, the muscle strength of the limbs returned to normal and the respiratory symptoms disappeared [41]. In a recently published article, two COVID-19 patients were diagnosed with Miller-Fisher syndrome (MFS) and multiple cranial neuritis, respectively [42]. These cases suggest a possible link between GBS and SARS-CoV-2 infection.

Some patients with severe COVID-19 progress rapidly and need to be transferred to an intensive care unit (ICU) for further treatment [43, 44]. In such patients, the peripheral nerves could be particularly susceptible to peripheral microcirculation disturbances, since the vessels supplying them with blood lack autoregulation [45]. ICU-acquired weakness, which can manifest as critical-illness polyneuropathy, critical-illness myopathy or both, is a frequent and disabling disorder in ICU patients [46]. Critical-illness polyneuropathy, an axonal sensory-motor polyneuropathy, is observed in up to a third of critically ill patients with systemic inflammatory response syndrome. Critical-illness myopathy, an acute

myopathy, develops in a similar setting, often in association with the use of corticosteroids and/or non-depolarizing neuromuscular-blocking agents [47].

Tsai et al. [35] presented data from four patients with probable SARS who developed axonal polyneuropathy, myopathy or both (2004). All of them had received intubation for respiratory distress and a high dose of steroid therapy for multiple organ failure. They developed distal-predominant weakness in all four limbs and a mild decrease in deep-tendon reflexes three to four weeks after the onset of SARS. The most likely diagnoses were critical-illness polyneuropathy and/or critical-illness myopathy. Some viruses, such as cytomegalovirus and varicella zoster virus, may cause peripheral neuropathy by directly attacking the nerves. It is not known whether direct attacks of the peripheral nervous system occur in HCoV-associated neuropathy.

Rhabdomyolysis

Rhabdomyolysis refers to the damage to striated muscle, the destruction of the muscle cell membrane integrity and the release of myoglobin, creatine kinase, other enzymes, small molecules and toxic substances into the systemic circulation due to various traumatic and non-traumatic factors, resulting in a group of clinical syndromes of organ damage [48, 49]. Clinical examination, history evaluation, laboratory studies, muscle biopsies and genetic testing are useful tools for diagnosing rhabdomyolysis and differentiating acquired from inherited cases. Acquired cases may be due to substance abuse, medication or toxic exposures, electrolyte abnormalities, endocrine disturbances and autoimmune myopathies [50].

In several recent studies on COVID-19 [5, 12, 20], a few patients exhibited varying degrees of myalgia, fatigue and elevated creatine and creatine kinase levels. In the study of Guan et al., two patients clearly developed rhabdomyolysis as a complication of COVID-19, while 14.90% (164/1099) exhibited myalgia or arthralgia symptoms and 13.7% (90/657) had creatine kinase levels ≥ 200 U/L [5]. Tong's research group reported that a patient diagnosed with COVID-19 had pain and weakness in both lower limbs and obvious tenderness after the ninth day of admission. Laboratory examination indicated that the patient's myoglobin level was >12,000.0 μg/L (reference 0-140 μg/L), creatine kinase was 11,842 U/L (reference 38-174 U/L) and lactate dehydrogenase was 2,347 U/L (reference 109-245 U/L). The authors added hydration, alkalization, plasma transfusion, gamma globulin and symptomatic support therapy based on the patient's previous treatment with oxygen, antivirals, antibiotics and methylprednisolone [51]. Creatine kinase and myoglobin are important indicators of rhabdomyolysis, but they are not routinely detected in the clinical practice. When patients have local muscle pain and weakness, rhabdomyolysis should be considered.

In previous SARS studies, some patients were clearly diagnosed with critical-illness myopathy [35] and rhabdomyolysis [50, 52]. In such patients, it cannot be ruled out that rhabdomyolysis may have developed due to the use of corticosteroids and/or nondepolarizing neuromuscular-blocking agents; however, the association of rhabdomyolysis with viruses such as influenza viruses A and B, human immunodeficiency virus, Coxsackie virus, cytomegalovirus, West Nile virus and dengue virus has also been well described [53–56]. Nevertheless, there is not yet sufficient evidence that HCoVs can directly invade muscle cells.

Acute cerebrovascular disease

The population is generally susceptible to SARS-CoV-2, but the elderly are more susceptible (the median age of hospitalized patients in one study was 56 years [interquartile range, 42-68 years; range, 22-92 years] [20]), and such patients are already at high risk for cerebrovascular diseases. Viral infections are known to be associated with an increased risk of stroke [57]. In a study by Mao et al., 214 patients diagnosed with COVID-19 were enrolled, and six (2.80%) of them developed acute cerebrovascular disease (five cases of ischemic stroke and one case of cerebral hemorrhage). All but one of these patients (an ischemic stroke patient) died of respiratory failure [13]. In a study of 206 SARS patients in Singapore, large artery stroke was diagnosed in five patients, of whom four were critically ill and three died [58]. Strokes are not uncommon in critically ill patients with multiple comorbidities, so SARS-CoV-2 infections in humans may increase the risk of stroke.

Central nervous system infection

Central nervous system infections are among the most critical problems in public health, as patients frequently exhibit neurologic sequelae. The clinical manifestations include a fever, headache, vomiting, stiff neck, afebrile seizures and status epilepticus. HCoVs cause a certain degree of nerve erosion, but their capacity to infect the central nervous system in humans has not been well characterized [10, 59]. Moriguchi et al. described a patient with SARS-CoV-2-associated meningitis who was brought to the hospital by ambulance due to convulsions and a coma. Interestingly, SARS-CoV-2 RNA was not detected in the patient's nasopharyngeal swab, but was detected in the patient's cerebrospinal fluid [60]. Zhao et al. [61] reported spinal cord involvement in a COVID-19 patient one week after the onset of fever. After admission, his SARS-CoV-2 RNA

nasopharyngeal swab test was positive. Based on the patient's acute flaccid myelitis of the lower limbs, urinary and bowel incontinence, and sensory level at T10, a diagnosis of acute myelitis was more likely. After the patient had been treated with high-flow oxygen, antiviral medication, steroids and human immunoglobulin, his body temperature returned to normal and two subsequent SARS-CoV-2 RNA nasopharyngeal swab tests were negative. The muscle strength of both upper limbs recovered to grade 4/5, while the muscle strength of both lower limbs was grade 1/5. This study indicated that acute myelitis may be a neurological complication of COVID-19. The above cases demonstrate the potential for neurological invasion of SARS-CoV-2.

The presence of HCoV in human central nervous system-related samples was detected as early as 1980 in autopsies of patients with multiple sclerosis [62]. In 2004, genetic material from SARS-CoV was detected in cerebrospinal fluid samples from a 32-year-old woman. The patient had a generalized tonic-clonic convulsions with loss of consciousness and up-rolling eyeballs lasting for one minute [38]. Another patient, a doctor infected with the SARS virus, had symptoms of restlessness, vomiting and confusion on the 33th day of illness. The patient died after treatment failed, and a brain biopsy was performed. A fragment specific for SARS HCoV was amplified from cultures of the brain suspension, and transmission electronic microscopy revealed the presence of an enveloped virus morphologically compatible with a coronavirus in the cultures [63]. Since some COVID-19 patients have complained of headaches, nausea etc, care providers should be alert for central nervous system infections caused by SARS-CoV-2 if such patients also exhibit symptoms such as a fever, epilepsy and disturbances of consciousness.

Mechanisms of nervous system damage due to SARS-CoV-2 infections

In this section, we will explore various mechanisms that may explain the correlation between COVID-19 and neurological disease.

Hypoxemia

In a clinical retrospective study of 138 people, the most common complication of COVID-19 during hospital admission was pneumonia, followed by acute respiratory distress syndrome (19.60%) and shock (8.70%) [20]. The patients in this study had varying degrees of hypoxia, accompanied by hypoxemia. Most patients received oxygen inhalation (ordinary oxygen inhalation, 106 [76.81%]), and many received mechanical ventilation (non-invasive ventilation, 15 [10.09%]; intermittent mandatory ventilation, 17 [12.32%]). More than 20% of the oxygen consumed by humans is used by the brain for ATP production to generate the required membrane potential [64]. As soon as anoxia sets in, ATP synthase begins to pump protons out of the mitochondrial matrix to maintain the mitochondrial membrane potential. Continued lack of oxygen can eventually lead to the loss of high-energy phosphate esters, disturbances of neurotransmitter metabolism, the breakdown of the membrane, the failure of mitochondria and the accumulation of intracellular Ca^{2+}. The immediate consequence is irreversible neurological damage and even neuronal death [64, 65].

Lack of oxygen increases the risk of stroke. For instance, the prolonged hypoxia of obstructive sleep apnea hypopnea syndrome can damage the sleep structure, increase blood pressure, reduce cerebral blood flow and promote microthrombosis and atherosclerosis, thus impacting the prognosis and recurrence of cerebral infarction [66, 67]. Mao et al. reported that six COVID-19 patients had acute cerebrovascular disease: five with severe infections (5/88) and one with a non-severe infection (1/126) (P=0.03) [13]. The symptoms of hypoxia in COVID-19 patients are very obvious, and critical patients need ventilator support. COVID-19 patients admitted to the ICU tend to be older and have a greater number of comorbid conditions (e.g., hypertension, diabetes, cardiovascular and cerebrovascular diseases) than those not admitted to the ICU [20]. This suggests that older age and these comorbidities may be risk factors for poor outcomes [68, 69].

ACE2

The metallopeptidase ACE2 has been confirmed to be the cell receptor for SARS-CoV-2, just as it is for SARS-CoV [70, 71]. However, SARS-CoV-2 cannot enter cells through other coronavirus receptors such as aminopeptidase N and dipeptidyl peptidase [71]. ACE2 is highly expressed not only in the alveolar type II cells of the lungs and the upper and stratified epithelial cells of the esophagus, but also in the absorptive enterocytes of the ileum and colon [72, 73]. The main physiological function of ACE2 is to catalyze the conversion of angiotensin II to angiotensin (1-7), with a vasodilator effect. In brain tissues, angiotensin (1-7) stimulates Mas receptors to promote angiogenesis, and also inhibits oxidative stress, prevents neuroinflammation, improves cerebral blood flow, suppresses apoptosis and protects cerebral blood vessels [74].

Enhancing the expression of ACE2 may be an important strategy for treating cardiovascular and cerebrovascular

diseases [75]. SARS-CoV-2 patients with cerebrovascular disease may be more likely to develop into severe patients with a higher risk of death, so more timely diagnosis is needed for such patients. ACEI and angiotensin II receptor blocker antihypertensive drugs may increase the expression of the ACE2 receptor [76]. In order to avoid aggravating SARS-CoV-2 infection symptoms, it is recommended that hypertensive patients on blood pressure control medications stop using ACEI and angiotensin II receptor blocker antihypertensive drugs, and instead use calcium channel blocker diuretic antihypertensive drugs [77].

Immunization

The responses of the immune system can be divided into innate immunity (also known as non-specific immunity) and adaptive immunity (also known as specific immunity, which can be further divided into humoral immunity and cellular immunity) [76, 78]. The immune mechanisms induced by SARS-CoV-2 are unclear. After SARS-CoV-2 enters the body through ACE2, host factors trigger an immune response against the virus. The virus induces natural immunity, phagocytosis and phagocytic cell death, thus damaging tissues and organs. In four clinical retrospective studies that clearly identified the diagnosis of COVID-19 [12, 19, 20, 79], the absolute value of lymphocytes in most patients was reduced. These findings suggest that SARS-CoV-2 mainly attacks lymphocytes, especially T lymphocytes, similar to SARS-CoV.

CD4+ T cells are well known to regulate or "assist" the functioning of other lymphocytes. CD8+ T cells are cytotoxic and can kill virus-infected cells [80]. Barton et al. [81] reported that in two autopsies of COVID-19 patients, immunohistochemistry revealed a small number of CD3+ T lymphocytes infiltrating the alveolar septum, while CD20+ B-lymphocytes were rare. CD8+ T cells were slightly more prevalent than CD4+ T cells, and CD68 detection revealed a few macrophages. Some studies have suggested that the substantial decrease in the total number of lymphocytes in coronavirus patients may indicate that the virus consumes many immune cells and inhibits cellular immune function [82, 83].

After an antigen enters the body, the corresponding antigen-specific B cells are activated, induced to proliferate and eventually stimulated to differentiate into plasma cells. These plasma cells then produce specific antibodies that can enter the body fluid and exert immune effects. It is widely accepted that immunoglobulin M (IgM) provides the first line of defense during viral infections, prior to the generation of adaptive, high-affinity IgG responses that are important for long-term immunity and immunological memory [84]. Li et al. successfully developed a rapid detection IgG-IgM combined antibody test kit for the diagnosis of COVID-19. The kit has a sensitivity of 88.66% and a specificity of 90.63%, and can detect the infection within 15 minutes [85]. After the rehabilitation of most patients with the novel coronavirus, the body will produce specific antibodies that can kill and eliminate the virus.

On February 8, 2020, with the Pneumonia Diagnosis and Treatment Program for Novel Coronavirus Infection (Trial Version 5) [86] as a guide, The First People's Hospital of Jiangxia District carried out the first phase of a new convalescent plasma treatment on three critically ill patients. After 12 to 24 hours of convalescent plasma therapy, the patients' laboratory examination results, clinical signs and symptoms improved significantly. Plasma therapy not only is safe and potentially effective, but also stimulates humoral immunity [87].

Most COVID-19 patients have a good prognosis, while a few patients have mild symptoms in the early stage and suddenly deteriorate in the later stage of the disease or during the recovery process. A large number of patients have exhibited a 'cytokine storm' (the rapid production of cytokines such as tumor necrosis factor alpha, interleukin-1, interleukin-6 and interferon gamma) due to the viral infection, which sometimes has progressed to acute respiratory distress syndrome and multiple organ failure [12, 19]. It is already known that HCoV can spread from the respiratory tract to the central nervous system through transneuronal and hematogenous routes, resulting in encephalitis and neurological diseases [88]. The invasion of the blood-brain barrier by the coronavirus can destroy vascular endothelial connections, leading to blood-brain barrier dysfunction and enhanced permeability [89]. When the virus invades the human brain, it triggers immune damage, causing brain damage and acute or chronic inflammation, thus creating a vicious cycle.

Inflammation

Several current retrospective clinical studies have described COVID-19 patients with abnormally low lymphocyte counts, Prolonged prothrombin times and significantly increased lactate dehydrogenase levels. Patients transferred to the ICU had significantly higher white blood cell and neutrophil counts than those not transferred to the ICU, as well as higher levels of D-dimer, creatine kinase and creatine [20]. The complications in severe cases have included rhabdomyolysis, shock, acute cardiac injury and acute kidney injury. Several mechanisms are thought to link

infections with acute vascular events, including the release of proinflammatory cytokines, the disruption of atherosclerotic plaques, physiological changes in the heart rate and vasoconstriction [90]. The inflammatory response in severe pneumonia is not limited to lung tissue; rather, the systemic inflammatory response is activated, and its amplification cascade impairs the function of distant organs [57, 91].

Hypercoagulability

Middle-aged and elderly patients account for the majority of COVID-19 patients (especially critically ill patients) with abnormally increased D-dimer levels, and such patients are more prone to embolic vascular events and cerebrovascular disease [20]. Umapathi et al. postulated that a hypercoagulable state predisposed a group of mainly critically ill SARS patients to large cerebral arterial thromboembolism [58]. Providers treating critically ill COVID-19 patients with underlying diseases such as hypertension, diabetes, cancer, etc. should be alert to the potential for hypercoagulability and regularly assess routine blood coagulation.

Ethics statement

Our research does not require an ethics statement.

CONCLUSIONS

SARS-CoV-2 infection may involve the nervous system, and may cause diseases such as poly-neuropathy, myopathy, cerebral infarction and central nervous system infections. Cerebral infarction is the second most common cause of death and the leading cause of adult disability worldwide. Patients with cerebrovascular diseases may face greater risks during infections, so it is necessary to strengthen protection to avoid infection, perform secondary prevention measures and monitor patients' symptoms and vital signs. During the period of high incidence of COVID-19, neurologists need to pay great attention to the treatment of patients, especially those whose first symptoms are neurological symptoms.

AUTHOR CONTRIBUTIONS

Xiaojia Tang, Peipei Liu and Yingzhu Chen conceived and designed the research. Xiaojia Tan wrote the manuscript, and all authors contributed to manuscript revision, read and approved the submitted version.

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CONFLICTS OF INTEREST

No potential conflicts of interest were reported by the authors.

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REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

2. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol. 2020; 5:536–44. https://doi.org/10.1038/s41564-020-0695-z PMID:32123347

3. WHO Director-General's opening remarks at the media briefing on COVID-19 – March 11, 2020. https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020.

4. https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200507covid-19-sitrep-108.pdf?sfvrsn=44cc8ed8_2.

5. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, et al., and China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020; 382:1708–1720. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

6. Sun P, Qie S, Liu Z, Ren J, Li K, Xi J. Clinical characteristics of 50 466 hospitalized patients with 2019-nCoV infection. J MED VIROL. 2020. https://doi.org/10.2139/ssrn.3539664

7. Nanshan Z. SARS diagnosis and treatment protocol. Natl Med J China. 2003; 83.

8. Lei C, Fuchun Z. Clinical analysis of 260 cases of SARS in GuangZhou. J Infect Dis. 2003; 21.

9. Zhong N. Management and prevention of SARS in China. Philos Trans R Soc Lond B Biol Sci. 2004; 359:1115–16. https://doi.org/10.1098/rstb.2004.1491 PMID:15306397

10. Desforges M, Le Coupanec A, Dubeau P, Bourgouin A, Lajoie L, Dubé M, Talbot PJ. Human coronaviruses and other respiratory viruses: underestimated opportunistic pathogens of the central nervous system? Viruses. 2019; 12:14. https://doi.org/10.3390/v12010014 PMID:31861926

11. Tsai LK, Hsieh ST, Chang YC. Neurological manifestations in severe acute respiratory syndrome. Acta Neurol Taiwan. 2005; 14:113–19. PMID:16252612

12. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

13. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, Chang J, Hong C, Zhou Y, Wang D, Miao X, Li Y, Hu B. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. JAMA Neurol. 2020; 77:1–9. https://doi.org/10.1001/jamaneurol.2020.1127 PMID:32275288

14. Corman VM, Lienau J, Witzenrath M. [Coronaviruses as the cause of respiratory infections]. Internist (Berl). 2019; 60:1136–45. https://doi.org/10.1007/s00108-019-00671-5 PMID:31455974

15. Luk HK, Li X, Fung J, Lau SK, Woo PC. Molecular epidemiology, evolution and phylogeny of SARS coronavirus. Infect Genet Evol. 2019; 71:21–30. https://doi.org/10.1016/j.meegid.2019.03.001 PMID:30844511

16. Nassar MS, Bakhrebah MA, Meo SA, Alsuabeyl MS, Zaher WA. Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection: epidemiology, pathogenesis and clinical characteristics. Eur Rev Med Pharmacol Sci. 2018; 22:4956–4961. https://doi.org/10.26355/eurrev_201808_15635 PMID:30070331

17. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579:270–73. https://doi.org/10.1038/s41586-020-2012-7 PMID:32015507

18. Ameli J. Communicable diseases and outbreak control. Turk J Emerg Med. 2016 (Suppl 1); 15:20–26. https://doi.org/10.5505/1304.7361.2015.19970 PMID:27437528

19. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

20. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

21. Riou J, Althaus CL. Pattern of early human-to-human transmission of Wuhan 2019 novel coronavirus (2019-nCoV), December 2019 to January 2020. Euro Surveill. 2020; 25:2000058. https://doi.org/10.2807/1560-7917.ES.2020.25.4.2000058 PMID:32019669

22. National Health Commission of the People's Republic of China. Diagnosis and treatment of the novel coronavirus pneumonia (Trial version 7) [D]. http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989/files/ce3e6945832a438eaae415350a8ce964.pdf. 2020.

23. Fathizadeh H, Maroufi P, Momen-Heravi M, Dao S, Köse Ş, Ganbarov K, Pagliano P, Esposito S, Kafil HS. Protection and disinfection policies against SARS-CoV-2 (COVID-19). Infez Med. 2020; 28:185–91. PMID:32275260

24. Yang C, Ma QY, Zheng YH, Yang YX. [Transmission routes of 2019-novel coronavirus (2019-nCoV)]. Zhonghua Yu Fang Yi Xue Za Zhi. 2020; 54:374–77. https://doi.org/10.3760/cma.j.cn112150-20200216-0016 PMID:32268644

25. Li LY, Wu W, Chen S, Gu JW, Li XL, Song HJ, Du F, Wang G, Zhong CQ, Wang XY, Chen Y, Shah R, Yang HM, Cai Q. Digestive system involvement of novel coronavirus infection: prevention and control infection from a gastroenterology perspective. J Dig Dis. 2020; 21:199–204. https://doi.org/10.1111/1751-2980.12862 PMID:32267098

26. Yeo C, Kaushal S, Yeo D. Enteric involvement of coronaviruses: is faecal-oral transmission of SARS-CoV-2 possible? Lancet Gastroenterol Hepatol. 2020; 5:335–37. https://doi.org/10.1016/S2468-1253(20)30048-0 PMID:32087098

27. Zhu H, Wang L, Fang C, Peng S, Zhang L, Chang G, Xia S, Zhou W. Clinical analysis of 10 neonates born to mothers with 2019-nCoV pneumonia. Transl Pediatr. 2020; 9:51–60. https://doi.org/10.21037/tp.2020.02.06 PMID:32154135

28. Yang Y, Lu Q, Liu M, Wang Y, Zhang A, Jalali N, Dean N, Longini I, Halloran ME, Xu B, Zhang X, Wang L, Liu W, Fang L. Epidemiological and clinical features of the 2019 novel coronavirus outbreak in China. bioRxiv. https://doi.org/10.1101/2020.02.10.20021675

29. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020; 367:1260–63. https://doi.org/10.1126/science.abb2507 PMID:32075877

30. Xu X, Chen P, Wang J, Feng J, Zhou H, Li X, Zhong W, Hao P. Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. Sci China Life Sci. 2020; 63:457–60. https://doi.org/10.1007/s11427-020-1637-5 PMID:32009228

31. Special Expert Group for Control of the Epidemic of Novel Coronavirus Pneumonia of the Chinese Preventive Medicine Association. [An update on the epidemiological characteristics of novel coronavirus pneumonia (COVID-19)]. Zhonghua Liu Xing Bing Xue Za Zhi. 2020; 41:139–44. https://doi.org/10.3760/cma.j.issn.0254-6450.2020.02.002 PMID:32057211

32. Han G, Zhou YH. Possibly critical role of wearing masks in general population in controlling COVID-19. J Med Virol. 2020; 10:1002. https://doi.org/10.1002/jmv.25886 PMID:32293711

33. Lin YH, Liu CH, Chiu YC. Google searches for the keywords of "wash hands" predict the speed of national spread of COVID-19 outbreak among 21 countries. Brain Behav Immun. 2020; 87:30–32. https://doi.org/10.1016/j.bbi.2020.04.020 PMID:32283286

34. Ma QX, Shan H, Zhang HL, Li GM, Yang RM, Chen JM. Potential utilities of mask-wearing and instant hand hygiene for fighting SARS-CoV-2. J Med Virol. 2020; 10:1002. https://doi.org/10.1002/jmv.25805 PMID:32232986

35. Tsai LK, Hsieh ST, Chao CC, Chen YC, Lin YH, Chang SC, Chang YC. Neuromuscular disorders in severe acute respiratory syndrome. Arch Neurol. 2004; 61:1669–73. https://doi.org/10.1001/archneur.61.11.1669 PMID:15534177

36. Engel WK. Intravenous immunoglobulin G is remarkably beneficial in chronic immune dysschwannian/dysneuronal polyneuropathy, diabetes-2 neuropathy, and potentially in severe acute respiratory syndrome. Acta Myol. 2003; 22:97–103. PMID:15088499

37. Zochodne DW. SARS, SIRS, and neurological disease. Arch Neurol. 2004; 61:1647–48. https://doi.org/10.1001/archneur.61.11.1647 PMID:15534173

38. Lau KK, Yu WC, Chu CM, Lau ST, Sheng B, Yuen KY. Possible central nervous system infection by SARS coronavirus. Emerg Infect Dis. 2004; 10:342–44. https://doi.org/10.3201/eid1002.030638 PMID:15030709

39. Sommer C, Geber C, Young P, Forst R, Birklein F, Schoser B. Polyneuropathies. Dtsch Arztebl Int. 2018; 115:83–90. https://doi.org/10.3238/arztebl.2018.083 PMID:29478436

40. Gwathmey KG, Pearson KT. Diagnosis and management of sensory polyneuropathy. BMJ. 2019; 365:l1108. https://doi.org/10.1136/bmj.l1108 PMID:31068323

41. Zhao H, Shen D, Zhou H, Liu J, Chen S. Guillain-Barré syndrome associated with SARS-CoV-2 infection: causality or coincidence? Lancet Neurol. 2020; 19:383–84. https://doi.org/10.1016/S1474-4422(20)30109-5 PMID:32246917

42. Gutiérrez-Ortiz C, Méndez A, Rodrigo-Rey S, San Pedro-Murillo E, Bermejo-Guerrero L, Gordo-Mañas R, de Aragón-Gómez F, Benito-León J. Miller Fisher syndrome and polyneuritis cranialis in COVID-19. Neurology. 2020; 95:e601–05. https://doi.org/10.1212/WNL.0000000000009619 PMID:32303650

43. Li T, Lu H, Zhang W. Clinical observation and management of COVID-19 patients. Emerg Microbes Infect. 2020; 9:687–90. https://doi.org/10.1080/22221751.2020.1741327 PMID:32208840

44. Jin YH, Cai L, Cheng ZS, Cheng H, Deng T, Fan YP, Fang C, Huang D, Huang LQ, Huang Q, Han Y, Hu B, Hu F, et al, and for the Zhongnan Hospital of Wuhan University

Novel Coronavirus Management and Research Team, Evidence-Based Medicine Chapter of China International Exchange and Promotive Association for Medical and Health Care (CPAM). A rapid advice guideline for the diagnosis and treatment of 2019 novel coronavirus (2019-nCoV) infected pneumonia (standard version). Mil Med Res. 2020; 7:4. https://doi.org/10.1186/s40779-020-0233-6 PMID:32029004

45. Witt NJ, Zochodne DW, Bolton CF, Grand'Maison F, Wells G, Young GB, Sibbald WJ. Peripheral nerve function in sepsis and multiple organ failure. Chest. 1991; 99:176–84. https://doi.org/10.1378/chest.99.1.176 PMID:1845860

46. Intiso D. ICU-acquired weakness: should medical sovereignty belong to any specialist? Crit Care. 2018; 22:1. https://doi.org/10.1186/s13054-017-1923-7 PMID:29301549

47. Bird SJ. Diagnosis and management of critical illness polyneuropathy and critical illness myopathy. Curr Treat Options Neurol. 2007; 9:85–92. https://doi.org/10.1007/s11940-007-0034-1 PMID:17298769

48. Nance JR, Mammen AL. Diagnostic evaluation of rhabdomyolysis. Muscle Nerve. 2015; 51:793–810. https://doi.org/10.1002/mus.24606 PMID:25678154

49. Huerta-Alardín AL, Varon J, Marik PE. Bench-to-bedside review: rhabdomyolysis — an overview for clinicians. Crit Care. 2005; 9:158–69. https://doi.org/10.1186/cc2978 PMID:15774072

50. Chen LL, Hsu CW, Tian YC, Fang JT. Rhabdomyolysis associated with acute renal failure in patients with severe acute respiratory syndrome. Int J Clin Pract. 2005; 59:1162–66. https://doi.org/10.1111/j.1368-5031.2005.00540.x PMID:16178983

51. Jin M, Tong Q. Rhabdomyolysis as potential late complication associated with COVID-19. Emerg Infect Dis. 2020; 26:1618–20. https://doi.org/10.3201/eid2607.200445 PMID:32197060

52. Wang JL, Wang JT, Yu CJ, Chen YC, Hsueh PR, Hsiao CH, Kao CL, Chang SC, Yang PC. Rhabdomyolysis associated with probable SARS. Am J Med. 2003; 115:421–22. https://doi.org/10.1016/s0002-9343(03)00448-0 PMID:14553890

53. Fröhlich G, Grawe A, Schmidt N, Roth R, Märker-Hermann E. [Difficult course of rhabdomyolysis in influenza A/H1N1]. Dtsch Med Wochenschr. 2013; 138:2351–54. https://doi.org/10.1055/s-0033-1349642 PMID:24193860

54. Verdolin LD, Borner AR, Mussi H, Gismondi RA, Schau B, Ramos RC. [Rhabdomyolysis associated with dengue fever in a lupic patient]. Rev Bras Reumatol. 2014; 54:318–21. https://doi.org/10.1016/j.rbr.2013.02.003 PMID:25627228

55. Shanmugam S, Seetharaman M. Viral rhabdomyolysis. South Med J. 2008; 101:1271–72. https://doi.org/10.1097/SMJ.0b013e318183649a PMID:19005459

56. de Kanter CT, Keuter M, van der Lee MJ, Koopmans PP, Burger DM. Rhabdomyolysis in an HIV-infected patient with impaired renal function concomitantly treated with rosuvastatin and lopinavir/ritonavir. Antivir Ther. 2011; 16:435–37. https://doi.org/10.3851/IMP1747 PMID:21555828

57. Warren-Gash C, Blackburn R, Whitaker H, McMenamin J, Hayward AC. Laboratory-confirmed respiratory infections as triggers for acute myocardial infarction and stroke: a self-controlled case series analysis of national linked datasets from Scotland. Eur Respir J. 2018; 51:1701794. https://doi.org/10.1183/13993003.01794-2017 PMID:29563170

58. Umapathi T, Kor AC, Venketasubramanian N, Lim CC, Pang BC, Yeo TT, Lee CC, Lim PL, Ponnudurai K, Chuah KL, Tan PH, Tai DY, Ang SP. Large artery ischaemic stroke in severe acute respiratory syndrome (SARS). J Neurol. 2004; 251:1227–31. https://doi.org/10.1007/s00415-004-0519-8 PMID:15503102

59. Bohmwald K, Gálvez NM, Ríos M, Kalergis AM. Neurologic alterations due to respiratory virus infections. Front Cell Neurosci. 2018; 12:386. https://doi.org/10.3389/fncel.2018.00386 PMID:30416428

60. Moriguchi T, Harii N, Goto J, Harada D, Sugawara H, Takamino J, Ueno M, Sakata H, Kondo K, Myose N, Nakao A, Takeda M, Haro H, et al. A first case of meningitis/encephalitis associated with SARS-coronavirus-2. Int J Infect Dis. 2020; 94:55–58. https://doi.org/10.1016/j.ijid.2020.03.062 PMID:32251791

61. Zhao K, Huang J, Dai D, Feng Y, Liu L, Nie S. Acute myelitis after SARS-CoV-2 infection_ a case report. medRxiv preprint. 2020. https://doi.org/10.1101/2020.03.16.20035105

62. Burks JS, DeVald BL, Jankovsky LD, Gerdes JC. Two coronaviruses isolated from central nervous system

tissue of two multiple sclerosis patients. Science. 1980; 209:933–34. https://doi.org/10.1126/science.7403860 PMID:7403860

63. Xu J, Zhong S, Liu J, Li L, Li Y, Wu X, Li Z, Deng P, Zhang J, Zhong N, Ding Y, Jiang Y. Detection of severe acute respiratory syndrome coronavirus in the brain: potential role of the chemokine mig in pathogenesis. Clin Infect Dis. 2005; 41:1089–96. https://doi.org/10.1086/444461 PMID:16163626

64. Chan JW, Bell KE. Global cerebral anoxia. Can J Neurol Sci. 2002; 29:88–90. https://doi.org/10.1017/s0317167100001797 PMID:11858543

65. Nathaniel TI, Williams-Hernandez A, Hunter AL, Liddy C, Peffley DM, Umesiri FE, Imeh-Nathaniel A. Tissue hypoxia during ischemic stroke: adaptive clues from hypoxia-tolerant animal models. Brain Res Bull. 2015; 114:1–12. https://doi.org/10.1016/j.brainresbull.2015.02.006 PMID:25738761

66. Koehler U, Cassel W, Hildebrandt O, Kesper K, Kianinejad P, Nell C, Mayer G, Ohl G. [Obstructive sleep apnea in neurological diseases: specially as a risk factor for stroke]. Nervenarzt. 2014; 85:35–42. https://doi.org/10.1007/s00115-013-3890-9 PMID:24362594

67. Losurdo A, Brunetti V, Broccolini A, Caliandro P, Frisullo G, Morosetti R, Pilato F, Profice P, Giannantoni NM, Sacchetti ML, Testani E, Vollono C, Della Marca G. Dysphagia and obstructive sleep apnea in acute, first-ever, ischemic stroke. J Stroke Cerebrovasc Dis. 2018; 27:539–46. https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.09.051 PMID:29074066

68. Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: origin, transmission, and characteristics of human coronaviruses. J Adv Res. 2020; 24:91–98. https://doi.org/10.1016/j.jare.2020.03.005 PMID:32257431

69. Wang B, Li R, Lu Z, Huang Y. Does comorbidity increase the risk of patients with COVID-19: evidence from meta-analysis. Aging (Albany NY). 2020; 12:6049–57. https://doi.org/10.18632/aging.103000 PMID:32267833

70. Kuhn JH, Li W, Choe H, Farzan M. Angiotensin-converting enzyme 2: a functional receptor for SARS coronavirus. Cell Mol Life Sci. 2004; 61:2738–43. https://doi.org/10.1007/s00018-004-4242-5 PMID:15549175

71. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CH, Chen HD, Chen J, Luo Y, et al. Discovery of a novel coronavirus associated with the recent pneumonia outbreak in humans and its potential bat origin. bioRxiv. 2020. https://doi.org/10.1101/2020.01.22.914952

72. Wang Z, Xu X. scRNA-seq profiling of human testes reveals the presence of the ACE2 receptor, a target for SARS-CoV-2 infection in spermatogonia, leydig and sertoli cells. Cells. 2020; 9:920. https://doi.org/10.3390/cells9040920 PMID:32283711

73. Tian Y, Rong L, Nian W, He Y. Review article: gastrointestinal features in COVID-19 and the possibility of faecal transmission. Aliment Pharmacol Ther. 2020; 51:843–51. https://doi.org/10.1111/apt.15731 PMID:32222988

74. Touyz RM, Li H, Delles C. ACE2 the janus-faced protein - from cardiovascular protection to severe acute respiratory syndrome-coronavirus and COVID-19. Clin Sci (Lond). 2020; 134:747–50. https://doi.org/10.1042/CS20200363 PMID:32255491

75. Santos RA, Sampaio WO, Alzamora AC, Motta-Santos D, Alenina N, Bader M, Campagnole-Santos MJ. The ACE2/Angiotensin-(1-7)/MAS axis of the renin-angiotensin system: focus on angiotensin-(1-7). Physiol Rev. 2018; 98:505–53. https://doi.org/10.1152/physrev.00023.2016 PMID:29351514

76. Brodin P. Powerful populations respond to viruses and vaccines. Immunity. 2015; 43:1035–37. https://doi.org/10.1016/j.immuni.2015.11.018 PMID:26682979

77. Expert consensus on the prevention and control of novel coronavirus infections in neurology (first edition). 2020. http://www.cnstroke.com/

78. Brodin P, Davis MM. Human immune system variation. Nat Rev Immunol. 2017; 17:21–29. https://doi.org/10.1038/nri.2016.125 PMID:27916977

79. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al. Clinical characteristics of 2019 novel coronavirus infection in China. medRxiv. https://doi.org/10.1101/2020.02.06.20020974

80. Cecere TE, Todd SM, Leroith T. Regulatory T cells in arterivirus and coronavirus infections: do they protect against disease or enhance it? Viruses. 2012; 4:833–46. https://doi.org/10.3390/v4050833 PMID:22754651

81. Pérez CA. Looking ahead: The risk of neurologic complications due to COVID-19. Neurol Clin Pract. 2020: 10:371-74.

https://doi.org/10.1212/CPJ.0000000000000836

82. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, Pan P, Wang W, Hu D, Liu X, Zhang Q, Wu J. Coronavirus infections and immune responses. J Med Virol. 2020; 92:424–32. https://doi.org/10.1002/jmv.25685 PMID:31981224

83. Racine R, Winslow GM. IgM in microbial infections: taken for granted? Immunol Lett. 2009; 125:79–85. https://doi.org/10.1016/j.imlet.2009.06.003 PMID:19539648

84. Li Z, Yi Y, Luo X, Xiong N, Liu Y, Li S, Sun R, Wang Y, Hu B, Chen W, Zhang Y, Wang J, Huang B, et al. Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. J Med Virol. 2020; 10:1002. https://doi.org/10.1002/jmv.25727 PMID:32104917

85. Pneumonia Diagnosis and Treatment Program for Novel Coronavirus Infection (Trial Version 5). Chin J Integr Med. 2020.

86. Bloch EM, Shoham S, Casadevall A, Sachais BS, Shaz B, Winters JL, van Buskirk C, Grossman BJ, Joyner M, Henderson JP, Pekosz A, Lau B, Wesolowski A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. J Clin Invest. 2020; 130:2757–65. https://doi.org/10.1172/JCI138745 PMID:32254064

87. Sun T, Guan J. Novel coronavirus and the central nervous system. Eur J Neurol. 2020; 27:e52-e52. https://doi.org/10.1111/ene.14227 PMID:32216009

88. Bleau C, Filliol A, Samson M, Lamontagne L. Brain Invasion by Mouse Hepatitis Virus Depends on Impairment of Tight Junctions and Beta Interferon Production in Brain Microvascular Endothelial Cells. J Virol. 2015; 89:9896–908. https://doi.org/10.1128/JVI.01501-15 PMID:26202229

89. Corrales-Medina VF, Madjid M, Musher DM. Role of acute infection in triggering acute coronary syndromes. Lancet Infect Dis. 2010; 10:83–92. https://doi.org/10.1016/S1473-3099(09)70331-7 PMID:20113977

90. Li H, Cao B. New antiviral drugs for coronavirus are expected in 2019. 2020.

91. Arabi YM, Harthi A, Hussein J, Bouchama A, Johani S, Hajeer AH, Saeed BT, Wahbi A, Saedy A, AlDabbagh T, Okaili R, Sadat M, Balkhy H. Severe neurologic syndrome associated with Middle East respiratory syndrome corona virus (MERS-CoV). Infection. 2015; 43:495–501. https://doi.org/10.1007/s15010-015-0720-y PMID:25600929

Review

SARS-CoV-2, immunosenescence and inflammaging: partners in the COVID-19 crime

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ABSTRACT

Pneumonia outbreak in the city of Wuhan, China, prompted the finding of a novel strain of severe acute respiratory syndrome virus (SARS-CoV-2). Here, we discuss potential long-term consequences of SARS-CoV-2 infection, and its possibility to cause permanent damage to the immune system and the central nervous system. Advanced chronological age is one of the main risk factors for the adverse outcomes of COVID-19, presumably due to immunosenescence and chronic low-grade inflammation, both characteristic of the elderly. The combination of viral infection and chronic inflammation in advanced chronological age might cause multiple detrimental unforeseen consequences for the predisposition and severity of neurodegenerative diseases and needs to be considered so that we can be prepared to deal with future outcomes of the ongoing pandemic.

INTRODUCTION

At the end of 2019, the novel severe acute respiratory syndrome coronavirus (SARS-CoV-2) emerged in Wuhan, China, as the causative agent of Coronavirus Disease 2019 (COVID-19) [1, 2]. The most prominent clinical symptom of COVID-19 is extensive lung damage, accompanied by respiratory distress of varying severity [3]. Within only 2-3 months, SARS-CoV-2 caused a worldwide health emergency and a pandemic, by infecting over 15 million people and, at the point of writing of this text, taking more than 633,000 lives. Within this short period, the pandemic has also triggered an avalanche of social and economic consequences that promise to continue growing, and that will scar our society [1].

SARS-CoV-2 belongs to the family of coronaviruses (CoV), together with SARS-CoV and Middle East respiratory syndrome CoV – two highly pathogenic viral strains that caused significant medical turmoil in the recent past and were responsible for considerable lethality [4]. The same family also includes several harmless viruses (HKU, 229E) [5]. The coronavirus family shares some overall similarities with the influenza A virus (IAV) H1N1 in the context of immune system activation, which includes allowing interferon-stimulated genes (ISG) effector response, responsible for the first defense against viral infection [6].

SARS-CoV-2 is a large and enveloped virus with positive-sense, single-stranded RNA genome [7]. The

infection is initiated by the binding of the viral spike (S) protein to ACE2 receptor at the host cell surface (Figure 1) [8], followed by the internalization and replication of the virus, culminating in the cell lysis and the exit of newly formed viral particles [9].

Although no treatment or preventive measures against SARS-CoV-2 exist at the present moment, the scientific community is working tirelessly, producing daily results on the molecular properties of the new virus and the plethora of its interaction with the host cells and tissues.

While at the clinical level, the respiratory problems are one of the main hallmarks of the disease, the molecular alterations among the severe cases of COVID-19 include signs of hyperinflammation characteristic of immunopathologies. The most striking example is a systemic inflammatory response known as cytokine release syndrome (or cytokine storm) due to massive T cell stimulation [10].

Here, we address the major clinical features of COVID-19 and discuss its potential effects on the aged population, from the perspective of its incidence and severity, as well as long-term effects in developing age-related diseases of the central nervous system.

On the one hand, aging affects the severity of COVID-19 and, on the other, is the leading risk factor for the development of neurodegenerative diseases [11]. Although the link between the SARS-CoV-2 and neurodegeneration has yet to be established, the cocktail of infection stress, chronic inflammation, and advanced chronological age may cause multiple detrimental unforeseen consequences to the risk and severity of neurodegenerative diseases. Therefore, it needs to be seriously considered so that we can be prepared to deal with future outcomes of the ongoing pandemic.

Clinical aspects of SARS-CoV-2 infection

The clinical spectrum associated with SARS-CoV-2 infection varies among the infected population depending on the time point of the diagnosis. At the moment of seeking medical attention, the most common symptoms are fever (>37.4°C), fatigue, dry cough, myalgia, and dyspnea [12]. The reduced ability to smell, or hyposmia, has been characterized as a major symptom in otherwise mild cases [13]. The other typical symptoms associated with a common viral upper respiratory infection, such as nasal congestion and rhinorrhea, are very uncommon (< 5%) [14, 15].

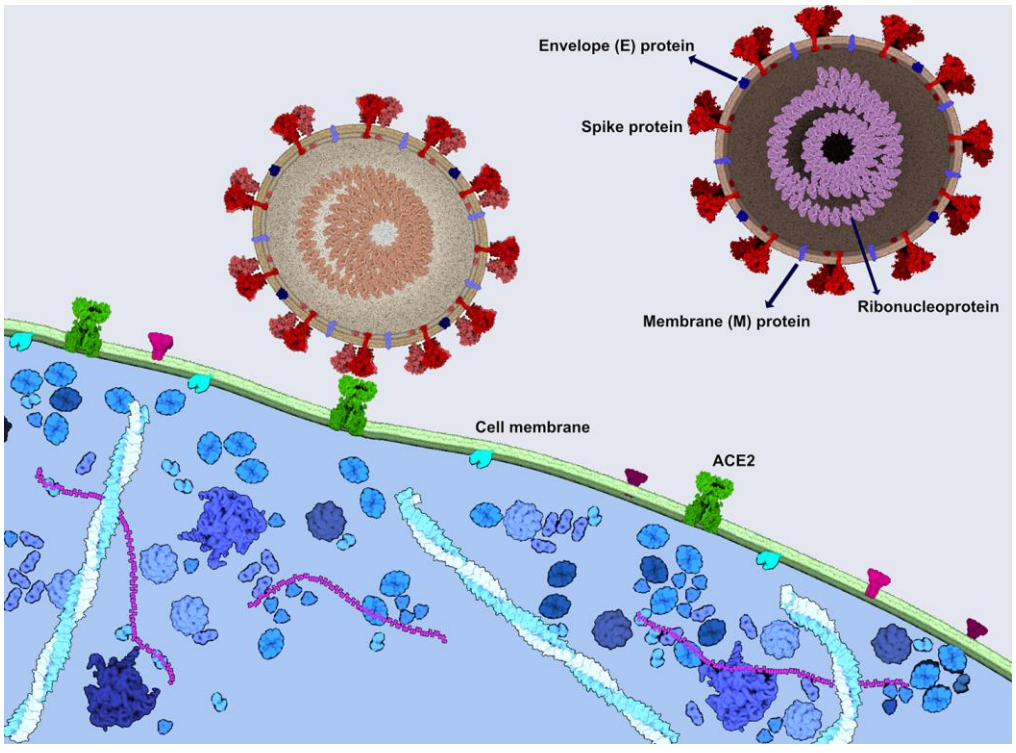

Figure 1. SARS-CoV-2 spike protein binds to the ACE2 receptor to enter the cells. Viral spike protein binds to the ACE2 receptor in the human cell membrane, followed by the internalization of the virus. SARS-CoV-2 consists also of the ribonucleoprotein, envelope protein and a membrane protein. The image was generated using CellPAINT Software [100].

The SARS-CoV-2 infection primarily affects adults, with fewer cases reported in children of 15 years or younger [15, 16]. The virus enters the host through the upper airway, and the viral load peaks at approximately day ten after the onset of symptoms [17]. The highest spread during the initial phase of the epidemic in Wuhan was observed as a human-to-human transmission among otolaryngologists [18]. Subsequent studies conducted on infected patients demonstrated high SARS-CoV-2 titers in the mucosa of the nasal and oral cavity [19], which represents the way SARS-CoV-2 enters the host, most readily transmitted by respiratory droplets and direct contact. The asymptomatic form of transmission may have contributed to the rapid spread of the disease [12], but there is still no scientific consensus regarding this mechanism [20–22].

A significant portion of patients infected with SARS-CoV-2 also shows neurological symptoms such as headache, nausea, and vomiting (<5%). Other described neurologic manifestations associated with SARS-CoV-2 infections are impaired consciousness and cerebro-vascular disease [15, 23]. The first case of meningitis/ encephalitis associated with SARS-CoV-2 infection was also recently reported [24].

SARS-CoV, a closely related virus, enters into human host cells mediated mainly by the angiotensin-converting enzyme 2 (ACE2) receptor, expressed in human airway epithelia and lung parenchyma, but also present in vascular endothelial cells, kidney cells, cells from the small intestine, and the brain (Figure 1) [25, 26]. Usually located on type I and II alveolar cells in the lung, the ACE2 receptor was also found to bind SARS-CoV-2 with an estimated binding affinity 10-20 times greater than the one of SARS-CoV [27]. The mechanism of entry into the host target cells, for both SARS-CoV and SARS-CoV-2, is warranted by the spike (S) protein [28, 29]. When attached to ACE2, the cellular transmembrane serine protease 2 (TMPRSS2) primes the spike protein to trigger the entry of the virus into the cell [19, 29]. Therefore, the spread of SARS-CoV-2 also depends on TMPRSS2 activity [29].

Neurotropism highlights the prerequisite of awareness towards SARS-CoV-2 entering the central nervous system. The neuroinvasive propensity of CoV has been documented for almost all of the β-CoV, including SARS-CoV [30], MERS-CoV [31], HCoV-229E [32] and HCoV-OC43 [23]. Evidence suggests that the virus might first invade peripheral nerve terminals, thus gaining access to the central nervous system via synapse-connected route [33, 34].

SARS-CoV-2: immunosenescence and increased severity among older adults

Epidemiological studies show that older adults are the most affected by this pandemic [35], rendering the chronological age a risk factor in COVID-19. Moreover, studies reveal the variable host resistance between patients from the same age groups.

Casualties in all age groups are also associated with pre-existing conditions such as reduced lung function, cardiovascular problems, and oncological disease spectrum. However, other factors might affect the outcome of patients with COVID-19 [36], such as variable genetic background and epigenetic pre-disposition. All these effectors converge at the level of immune system attenuation.

Since the beginning of the SARS-CoV-2 outbreak, parallels were made with the influenza A virus H1N1 infection, due to its contributions to the mortality of the elderly. Influenza remains a serious global health threat that impacts all countries, with 290,000-750,000 influenza-related respiratory deaths worldwide every year [37].

Senescence defines a stable growth arrest induced when cells reach the end of their replicative potential or are exposed to various stressors, such as infection. Senescent cells accumulate in aging tissues and contribute to the development of age-related disorders [38]. However, it was only in 2011 when evidence was presented showing that the clearance of senescent cells can delay aging-associated diseases [39]. This discovery confirmed senescence as a hallmark of aging.

Like other tissues, the immune system is characterized by the decline of its functions with age (immuno-senescence), reflected not only in increased cancer prevalence, autoimmune and other chronic diseases but also in greater susceptibility to infections [40]. Understood as a gradual deterioration of the immune system brought on by natural age advancement, immunosenescence originates as a disability of T Cells (CD4 as well as CD8 positive) to function correctly [41].

Senescence compromises the ability of CD4+ T cells to correctly activate, differentiate, proliferate, and respond to the H1N1 virus [42]. Aged CD4+ T cells accumulate intrinsic defects that contribute to a reduced helper function during influenza infection [43, 44]. In vivo studies conducted on senescent mice have evidenced low H1N1 influenza-specific antibody titers after influenza infection that reflects the age-related lowered immune response [44].

Viral infections are also known as stressors that can induce senescence in different cell lines. The Dengue virus can cause senescence in endothelial cells [45], and the Measles virus leads to cellular senescence in normal and cancer fibroblasts [46]. Senescent cells can play a role during viral infection by limiting the proliferation of damaged cells. In fact, these cells help to control the viral replication, while in experimental studies, senescence induction restricts the infection in mice [47]. Moreover, the NS1 protein of the avian influenza H7N9 virus can induce growth arrest and cellular senescence in Neuro2a cells [48]. Neurons infected with influenza A virus can respond to the infection by producing oxygen radicals and nitric oxide (NO) [49]. NS1 protein leads to an increased release of NO in Neuro2a cells which causes a reduced proliferation, enlarged cell morphology, an up-regulation of IL-6 and IL-8 as well as increased SA-β-gal activity, all features of senescent cells [48].

Immunosenescence offers insights into the differential resistance of young vs. old individuals, as well as men vs. women, to SARS-CoV-2 infection [50]. The depletion of B lymphocyte-driven acquired immunity is a characteristic of old age, affecting predominantly men [51]. Aging diminishes the upregulation molecules essential for T cell priming and also reduces antiviral interferon (IFN) production by alveolar macrophages and dendritic cells (DCs) [52].

In summary, impairment in number, function, and activation of cells involved in the immune response [53–55] and aging of hematopoietic stem cells [56] are major phenotypes of the immune system associated with immunosenescence (Figure 2). Ultimately, these changes lead to a process termed "inflammaging," where low-grade inflammation is present at an advanced age and is associated with a worsening of chronic progressive medical conditions, such as congestive heart failure [57], and the onset of age-related diseases involving the central nervous system (e.g., Alzheimer's disease) [58]. When the age-associated inflammation persists in the long-term, it may lead to oxidative stress in various tissues, while also triggering organelle dysfunction (e.g., mitochondrial and lysosomal), which could, in turn, increase the cell vulnerability to infection.

Inflammaging: an ally of SARS-CoV-2

An age-related decline in cellular repair mechanisms causes accumulation of damage at genome and proteome levels. This can lead to systemic changes in the immune system and increase pro-inflammatory cytokine production (interferon, interleukin, etc.), resulting in inflammaging [57]. The increase in cytokine production originates from the tissue macrophages, which initiate and regulate the inflammation [59]. Macrophages may, therefore, play significant roles in inflammaging. Some of the cellular hallmarks of aging, such as deregulated nutrient signaling and mitochondrial dysfunction, are also implicated in inflammaging, thus promoting the inflammatory environment [60].

Macrophages are also affected by aging, characterized mainly by the reduced potential for phagocytosis, and a decline in the gut barrier function [61]. Alveolar macrophages (AM) maintain lung homeostasis and play an important role in the influenza infection [62]. In particular, aged AM have a reduced power to control lung damage during influenza infection. During the progress of aging, the number of AM is reduced, leading to a lowered ability for phagocytosis [63]. Previous studies have also shown a decline of innate immune receptor functions and a substantial increase in viral replication efficiency after influenza infection in aged or senescent cells [64]. While the detailed mechanisms remain to be further studied, a reduction of the interferon (IFN) response in senescent cells after

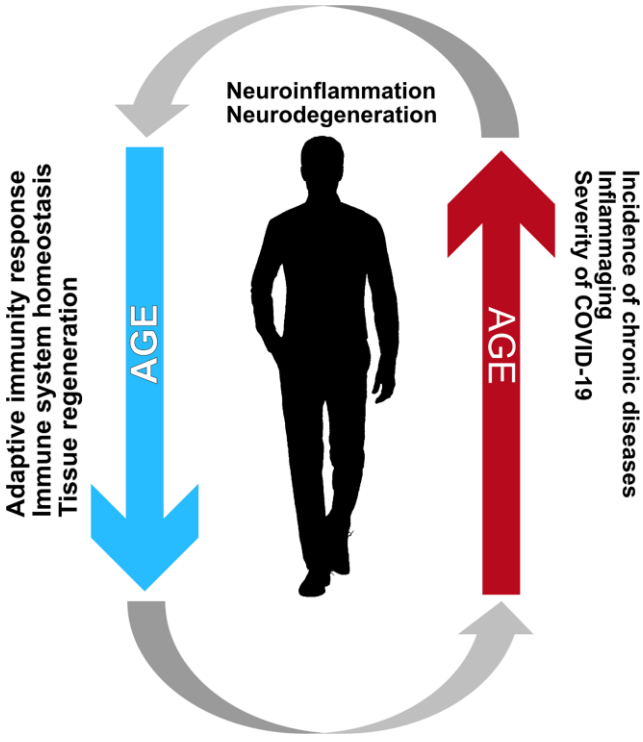

Figure 2. Immunosencescence and inflammaging create a vicious cycle creating an environment favorable for the development of neurodegenerative diseases. Such a relationship between these processes is mainly characteristic of the elderly and is the most likely reason for the increased incidence and adversity of COVID-19 among the elderly.

viral infection may play an important role. Moreover, a significant decrease in percentages and numbers of CD8+ T cells specific for at least one of the dominant epitopes of the influenza virus (influenza A nucleoprotein, NP, epitope) is typical for aged mice [65].

Pro-inflammatory cytokines play an important role in aging processes. The activation and the high levels of inflammatory cytokines such as IL-1, IL-6, TNF, and IFN-gamma are linked with morbidity and mortality in older patients [66]. In particular, IL-6 is a multi-functional cytokine produced in response to tissue damage and infections by multiple cell types [67]. Previous studies demonstrate its critical role in promoting lung tissue inflammation [68] and stimulating viral replication [69]. Moreover, elevated IL-6 is correlated with respiratory failure [10], and high concentrations of IL-6 in the serum is considered one of the hallmarks of severe MERS-CoV infections [70]. Additionally, an increase of IL-6 levels predicts adverse outcomes of COVID-19, underscoring inflammaging as the main ally of SARS-CoV-2 [35, 71]. Moreover, a recent study investigated the occurrence of cytokine storm in COVID-19 patients, also focusing on immunological characteristics of the response to COVID-19. In both mild and severe cases of COVID-19, increased levels of IL-6 are typical, while this is not the case among asymptomatic patients [10].

Inflammaging is also consistent with the gender bias of SARS-CoV-2. The more robust age-dependent activation of the innate pro-inflammatory pathways in COVID-19 is demonstrated in men compared to women [51], which is consistent with a higher rate of inflammaging among men [72]. A different situation among centenarians lends further support to the inflammaging importance for COVID-19 progression. Distinct longevity traits characterize centenarians, anti-inflammatory markers being the most prominent example, likely protecting them against the adverse outcomes of sustained inflammation as well as from the most severe forms of COVID-19 [73, 74].

Another critical factor is the impact of senescence in the lungs. Although COVID-19 shows symptoms across the entire body, the most prominent symptoms are respiratory and those associated with respiratory illness. The lung function tends to decrease with age having decreased alveolar elasticity [75], and increased senescence of epithelial cells and fibroblasts render cells frail to injuries such as the one caused by age-associated inflammation and viral infection [76]. Resident immune cells, most notably neutrophils, are also present in the lungs and are subject to immunosenescence. These cells become less functional due to age-associated chronic exposure to inflammatory cytokines [77], ultimately leading to fibrosis and aberrant tissue regeneration. The senescence phenotype, however, can be controlled by external factors, such as smoking [78], thus increasing the pool variability found in patients from the same age. In summary, the literature reviewed above may hold the key as to why the combination of immunosenescence and inflammaging does not allow an efficient response to the invasion of SARS-CoV-2 and why older individuals with co-morbidity are more prone to adverse outcomes of COVID-19 [79].

Diminished immune functions characterize immunosenescence, and inflammaging leads to a lack of anti-inflammatory modulators. The existing evidence suggests that inflammaging and immunosenescence, taken together, have vital roles in the decline of immune system functions to fight SARS-CoV-2 infection and lead to severe COVID-19 in older subjects (Figure 2).

SARS-CoV-2: a possible tipping point for inflammaging and neurodegeneration

Aging is the most significant risk factor for the development of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease, or amyotrophic lateral sclerosis (ALS). In PD, inflammation in the central nervous system (CNS), i.e., neuroinflammation, plays a vital role in the severity of the pathogenesis and is considered a key player in nigral cell loss [80].

Neuroinflammation is mainly regulated by glial cells, such as microglia and astrocytes. Microglia are considered the resident macrophages of the brain, therefore representing the first line of immune defense in the CNS. Moreover, they perform clearance of the metabolic waste, damaged cells, and pathogens, thus regulating both the pro-inflammatory and anti-inflammatory response [81]. During pathogenesis, microglia become activated due to cellular damage and the presence of protein aggregates in their surroundings, triggering the production of chemokines and cytokines such as TNF-α, IL-6, IL-1β, IFN-γ and CCL2 [82]. The resulting oxidative stress amplifies the damage to cellular components and further activates neighboring glial cells, thus causing a chronic activation [83]. Moreover, recent studies show that microglia can play a crucial role in defense of olfactory neuronal cells against viral infection [84]. Although data regarding the role of chemokines in SARS-CoV-2 infection is still scarce, it is known that infected epithelial cells upregulate genes encoding multiple chemokines such as CXCL1, CXCL3, CXCL6, CXCL16, and CXCL1. This increases the immune activation and recruitment of immune cells to the infected tissue, thus representing a potential therapeutic target [85].

It has been long established that peripheral inflammation associated with chronic diseases increases the production of cytokines, in particular IL-1β, in the CNS [86]. However, viral infections, such as with H1N1, can cause microglial activation [87]. This, in turn, increases the risk of developing diseases such as PD [88] and may trigger protein aggregation [89]. Another pointer towards neuro-immune crosstalk in neurodegeneration is the fact that nonsteroidal anti-inflammatory drugs also show a protective effect in the case of neurodegenerative diseases [90].

A milestone in the research on mechanisms of neuro-immune crosstalk was the discovery of the brain meningeal lymphatic system that clears proteins and metabolic waste from the cerebrospinal fluid (CSF) [91]. During aging, the lymphatic system becomes impaired due to a reduction in the lymphatic vessel diameter and leads to an increase in waste accumulation in the brain [92]. Such CNS-derived antigens contribute to the neuroinflammatory conditions, and their clearance is essential to counter the inflammation [91]. It is possible that due to peripheral inflammation, not only blood-borne cytokines can enter the brain, causing the detrimental neuroinflammatory effects, but also the immune cells present in the lymphatic system, exposing the brain to a vicious circle increasing its vulnerability to additional injuries.

The available literature on SARS-CoV-2 suggests that the virus may enter the nervous system via the lymphatic circulation [93]. SARS-CoV-2 can infect lymph endothelial cells [94] and, therefore, may use the paranasal lymph vessels to reach the brain. The presence of the virus was confirmed in the neuronal and capillary cells in the frontal lobe of the COVID-19 patients [95], associated with a worsening of neurological symptoms. The convergence of viral load in the nervous system and its relationship with brain lymphatics and microglial reaction against the virus may explain why some patients have prominent neurological symptoms, while others do not appear to experience these at all.

Aging triggers debilitating conditions, such as systemic low-grade inflammation and neurodegeneration. Such conditions can be set off or aggravated by viral infections, as evidenced by the H1N1 infection shown to contribute to PD development. The severity of SARS-CoV-2 infection indicates not only an overwhelming response of the immune system, but the presence of neurological symptoms suggests the connection with the CNS.

Severe neurological symptoms associated with COVID-19 have become increasingly noticeable after SARS-CoV-2 has been detected in the CSF of some patients [24]. A growing number of cases show neurological manifestations in COVID-19 patients, including examples of cerebrovascular disease, Guillain-Barré syndrome, encephalitis, and necrotizing encephalopathy [96]. The neurological symptoms appear in proportion with the severity of SARS-CoV-2 infection: patients with severe cases of COVID-19 show neurological manifestations (45.5%) with a higher incidence relative to the mild cases [97, 98]. The overall number of patients who displayed neurological symptoms is still low compared to respiratory manifestations. Still, the continuing pandemic and the data collected so far predict an increase in the number of neurological diseases that should not be underestimated [98]. It has also been proposed that SARS-CoV-2 infection may disrupt cellular homeostasis, ultimately leading to protein misfolding and, this way, increasing the propensity for the future development of neurodegenerative diseases [99].

This relationship calls for caution and extensive research related to the development of neuro-inflammation and neurodegenerative diseases among COVID-19 survivors.

CONCLUDING REMARKS

Our understanding of COVID-19 is growing by the day due to the increasing amount of clinical data and laboratory studies. The most prominent symptoms are associated with the tissues expressing the ACE2 receptor (airway epithelia and lung parenchyma). Still, the presence of neurological symptoms draws attention to the potential interaction of COVID-19 with the CNS.

Older people and people with co-morbidities are more prone to display severe symptoms of COVID-19 due to cellular senescence in the affected tissues and the immune system. Therefore, in the elderly, SARS-CoV-2 'preys' on the tissue debility and the deficiency of the immune system. The knowledge of immunosenescence and inflammaging provides a potential interpretation of epidemiological data underscoring the elderly as the population most sensitive to COVID-19.

Peripheral inflammation associated with aging and chronic diseases increases the production of cytokines also in the CNS. Similar effects can be triggered by viral infection via microglia activation, promoting protein aggregation, and, in turn, increasing the risk of developing neurodegenerative diseases [99]. Therefore, understanding the triangle between SARS-CoV2, immunosenescence, and inflammaging may shed important light on the molecular underpinnings of COVID-19, and open novel avenues for therapeutic

interventions. These are desperately needed so that our lives can return to the 'normality' we used to know before this pandemic.

AUTHOR CONTRIBUTIONS

All authors contributed in conceiving, discussion and writing of this manuscript.

CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

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REFERENCES

1. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY, Yuan ML, Zhang YL, Dai FH, et al. Author correction: a new coronavirus associated with human respiratory disease in China. Nature. 2020; 580:E7. https://doi.org/10.1038/s41586-020-2202-3 PMID:32296181

2. Chen X, Yu B. First two months of the 2019 coronavirus disease (COVID-19) epidemic in China: real-time surveillance and evaluation with a second derivative model. Glob Health Res Policy. 2020; 5:7. https://doi.org/10.1186/s41256-020-00137-4 PMID:32158961

3. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

4. de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. Nat Rev Microbiol. 2016; 14:523–34. https://doi.org/10.1038/nrmicro.2016.81 PMID:27344959

5. Su S, Wong G, Shi W, Liu J, Lai AC, Zhou J, Liu W, Bi Y, Gao GF. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. Trends Microbiol. 2016; 24:490–502. https://doi.org/10.1016/j.tim.2016.03.003 PMID:27012512

6. Menachery VD, Eisfeld AJ, Schäfer A, Josset L, Sims AC, Proll S, Fan S, Li C, Neumann G, Tilton SC, Chang J, Gralinski LE, Long C, et al. Pathogenic influenza viruses and coronaviruses utilize similar and contrasting approaches to control interferon-stimulated gene responses. mBio. 2014; 5:e01174–14. https://doi.org/10.1128/mBio.01174-14 PMID:24846384

7. Satija N, Lal SK. The molecular biology of SARS coronavirus. Ann N Y Acad Sci. 2007; 1102:26–38. https://doi.org/10.1196/annals.1408.002 PMID:17470909

8. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, Guo L, Guo R, Chen T, Hu J, Xiang Z, Mu Z, Chen X, et al. Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV. Nat Commun. 2020; 11:1620. https://doi.org/10.1038/s41467-020-15562-9 PMID:32221306

9. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol Biol. 2015; 1282:1–23. https://doi.org/10.1007/978-1-4939-2438-7_1 PMID:25720466

10. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, Wang T, Zhang X, Chen H, Yu H, Zhang X, Zhang M, Wu S, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest. 2020; 130:2620–29. https://doi.org/10.1172/JCI137244 PMID:32217835

11. Hou Y, Dan X, Babbar M, Wei Y, Hasselbalch SG, Croteau DL, Bohr VA. Ageing as a risk factor for neurodegenerative disease. Nat Rev Neurol. 2019; 15:565–81. https://doi.org/10.1038/s41582-019-0244-7 PMID:31501588

12. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

13. Lüers JC, Klußmann JP, Guntinas-Lichius O. [The COVID-19 pandemic and otolaryngology: what it comes down to?]. Laryngorhinootologie. 2020; 99:287–91. https://doi.org/10.1055/a-1095-2344 PMID:32215896

14. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7

PMID:32007143

15. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

16. Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, Ren R, Leung KS, Lau EH, Wong JY, Xing X, Xiang N, Wu Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. N Engl J Med. 2020; 382:1199–207. https://doi.org/10.1056/NEJMoa2001316 PMID:31995857

17. Lipsitch M, Cohen T, Cooper B, Robins JM, Ma S, James L, Gopalakrishna G, Chew SK, Tan CC, Samore MH, Fisman D, Murray M. Transmission dynamics and control of severe acute respiratory syndrome. Science. 2003; 300:1966–70. https://doi.org/10.1126/science.1086616 PMID:12766207

18. Lu D, Wang H, Yu R, Yang H, Zhao Y. Integrated infection control strategy to minimize nosocomial infection of coronavirus disease 2019 among ENT healthcare workers. J Hosp Infect. 2020; 104:454–55. https://doi.org/10.1016/j.jhin.2020.02.018 PMID:32114056

19. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, Yu J, Kang M, Song Y, Xia J, Guo Q, Song T, He J, et al. SARS-CoV-2 Viral Load in Upper Respiratory Specimens of Infected Patients. N Engl J Med. 2020; 382:1177–1179. https://doi.org/10.1056/NEJMc2001737 PMID:32074444

20. Aylward, Bruce (WHO); Liang W (PRC). Report of the WHO-China Joint Mission on Coronavirus Disease 2019 (COVID-19). WHO-China Jt Mission Coronavirus Dis 2019. 2020.

21. Yang H, Wu Z, McGoogan JM, World Health Organisation, Wölfel R, Corman VM, Guggemos W, Seilmaier M, Müller MA, Niemeyer D, Kelly TCJ, Vollmar P, Hoelscher M, et al. Factsheet for health professionals on Coronaviruses. Jama. 2020.

22. Day M. Covid-19: four fifths of cases are asymptomatic, China figures indicate. BMJ. 2020; 369:m1375. https://doi.org/10.1136/bmj.m1375 PMID:32241884

23. Dubé M, Le Coupanec A, Wong AH, Rini JM, Desforges M, Talbot PJ. Axonal transport enables neuron-to-neuron propagation of human coronavirus OC43. J Virol. 2018; 92:e00404–18. https://doi.org/10.1128/JVI.00404-18 PMID:29925652

24. Moriguchi T, Harii N, Goto J, Harada D, Sugawara H, Takamino J, Ueno M, Sakata H, Kondo K, Myose N, Nakao A, Takeda M, Haro H, et al. A first case of meningitis/encephalitis associated with SARS-coronavirus-2. Int J Infect Dis. 2020; 94:55–58. https://doi.org/10.1016/j.ijid.2020.03.062 PMID:32251791

25. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol. 2004; 203:631–37. https://doi.org/10.1002/path.1570 PMID:15141377

26. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res. 2000; 87:E1–9. https://doi.org/10.1161/01.res.87.5.e1 PMID:10969042

27. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020; 367:1260–63. https://doi.org/10.1126/science.abb2507 PMID:32075877

28. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature. 2003; 426:450–54. https://doi.org/10.1038/nature02145 PMID:14647384

29. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020; 181:271–80.e8. https://doi.org/10.1016/j.cell.2020.02.052 PMID:32142651

30. Glass WG, Subbarao K, Murphy B, Murphy PM. Mechanisms of host defense following severe acute respiratory syndrome-coronavirus (SARS-CoV) pulmonary infection of mice. J Immunol. 2004; 173:4030–39. https://doi.org/10.4049/jimmunol.173.6.4030 PMID:15356152

31. Li K, Wohlford-Lenane C, Perlman S, Zhao J, Jewell AK, Reznikov LR, Gibson-Corley KN, Meyerholz DK, McCray PB Jr. Middle east respiratory syndrome coronavirus causes multiple organ damage and lethal disease in

mice transgenic for human dipeptidyl peptidase 4. J Infect Dis. 2016; 213:712–22. https://doi.org/10.1093/infdis/jiv499 PMID:26486634

32. Talbot PJ, Ekandé S, Cashman NR, Mounir S, Stewart JN. Neurotropism of human coronavirus 229E. Adv Exp Med Biol. 1993; 342:339–46. https://doi.org/10.1007/978-1-4615-2996-5_52 PMID:8209751

33. Li YC, Bai WZ, Hirano N, Hayashida T, Hashikawa T. Coronavirus infection of rat dorsal root ganglia: ultrastructural characterization of viral replication, transfer, and the early response of satellite cells. Virus Res. 2012; 163:628–35. https://doi.org/10.1016/j.virusres.2011.12.021 PMID:22248641

34. Li YC, Bai WZ, Hirano N, Hayashida T, Taniguchi T, Sugita Y, Tohyama K, Hashikawa T. Neurotropic virus tracing suggests a membranous-coating-mediated mechanism for transsynaptic communication. J Comp Neurol. 2013; 521:203–12. https://doi.org/10.1002/cne.23171 PMID:22700307

35. Ruan Q, Yang K, Wang W, Jiang L, Song J. Correction to: clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Med. 2020; 46:1294–97. https://doi.org/10.1007/s00134-020-06028-z PMID:32253449

36. Madjid M, Safavi-Naeini P, Solomon SD, Vardeny O. Potential effects of coronaviruses on the cardiovascular system: a review. JAMA Cardiol. 2020. [Epub ahead of print]. https://doi.org/10.1001/jamacardio.2020.1286 PMID:32219363

37. FluNet, FluID, WHO Regional offices. WHO | Influenza update - 335. WHO. 2019.

38. Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, Carter C, Yu BP, Leeuwenburgh C. Molecular inflammation: underpinnings of aging and age-related diseases. Ageing Res Rev. 2009; 8:18–30. https://doi.org/10.1016/j.arr.2008.07.002 PMID:18692159

39. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature. 2011; 479:232–36. https://doi.org/10.1038/nature10600 PMID:22048312

40. Gavazzi G, Herrmann F, Krause KH. Aging and infectious diseases in the developing world. Clin Infect Dis. 2004; 39:83–91. https://doi.org/10.1086/421559 PMID:15206058

41. Kim JA, Seong RK, Shin OS. Enhanced viral replication by cellular replicative senescence. Immune Netw. 2016; 16:286–95. https://doi.org/10.4110/in.2016.16.5.286 PMID:27799874

42. Haynes L, Eaton SM. The effect of age on the cognate function of CD4+ T cells. Immunol Rev. 2005; 205:220–28. https://doi.org/10.1111/j.0105-2896.2005.00255.x PMID:15882356

43. Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. J Exp Med. 2004; 200:1613–22. https://doi.org/10.1084/jem.20041395 PMID:15611289

44. Lefebvre JS, Masters AR, Hopkins JW, Haynes L. Age-related impairment of humoral response to influenza is associated with changes in antigen specific T follicular helper cell responses. Sci Rep. 2016; 6:25051. https://doi.org/10.1038/srep25051 PMID:27109638

45. Shu MH, Wong PF, Johari J, Abubakar S. Dengue virus infection induces endothelial cells senescence. Int J Infect Dis. 2014; 21:224. https://doi.org/10.1016/j.ijid.2014.03.888

46. Chuprin A, Gal H, Biron-Shental T, Biran A, Amiel A, Rozenblatt S, Krizhanovsky V. Cell fusion induced by ERVWE1 or measles virus causes cellular senescence. Genes Dev. 2013; 27:2356–66. https://doi.org/10.1101/gad.227512.113 PMID:24186980

47. Baz-Martínez M, Da Silva-Álvarez S, Rodríguez E, Guerra J, El Motiam A, Vidal A, García-Caballero T, González-Barcia M, Sánchez L, Muñoz-Fontela C, Collado M, Rivas C. Cell senescence is an antiviral defense mechanism. Sci Rep. 2016; 6:37007. https://doi.org/10.1038/srep37007 PMID:27849057

48. Yan Y, Du Y, Zheng H, Wang G, Li R, Chen J, Li K. NS1 of H7N9 influenza a virus induces NO-mediated cellular senescence in Neuro2a cells. Cell Physiol Biochem. 2017; 43:1369–80. https://doi.org/10.1159/000481848 PMID:28992616

49. Akaike T, Maeda H. Nitric oxide and virus infection. Immunology. 2000; 101:300–08. https://doi.org/10.1046/j.1365-2567.2000.00142.x PMID:11106932

50. Prattichizzo F, Bonafè M, Giuliani A, Costantini A, Storci G, Sabbatinelli J, Olivieri F. Response to: letter to the editor on “Bonafè M, Prattichizzo F, Giuliani A, Storci G, Sabbatinelli J, Olivieri F. Inflamm-aging: why older men are the most susceptible to SARS-CoV-2 complicated outcomes. Cytokine growth factor rev” by Eugenia

Quiros-Roldan, Giorgio Biasiotto and Isabella Zanella. Cytokine Growth Factor Rev. 2020; S1359-6101:30189–81. https://doi.org/10.1016/j.cytogfr.2020.07.013 PMID:32718829

51. Márquez EJ, Chung CH, Marches R, Rossi RJ, Nehar-Belaid D, Eroglu A, Mellert DJ, Kuchel GA, Banchereau J, Ucar D. Sexual-dimorphism in human immune system aging. Nat Commun. 2020; 11:751. https://doi.org/10.1038/s41467-020-14396-9 PMID:32029736

52. Kovacs EJ, Boe DM, Boule LA, Curtis BJ. Inflammaging and the lung. Clin Geriatr Med. 2017; 33:459–71. https://doi.org/10.1016/j.cger.2017.06.002 PMID:28991644

53. Toapanta FR, Ross TM. Impaired immune responses in the lungs of aged mice following influenza infection. Respir Res. 2009; 10:112. https://doi.org/10.1186/1465-9921-10-112 PMID:19922665

54. Nogusa S, Ritz BW, Kassim SH, Jennings SR, Gardner EM. Characterization of age-related changes in natural killer cells during primary influenza infection in mice. Mech Ageing Dev. 2008; 129:223–30. https://doi.org/10.1016/j.mad.2008.01.003 PMID:18304606

55. Jiang J, Fisher EM, Murasko DM. CD8 T cell responses to influenza virus infection in aged mice. Ageing Res Rev. 2011; 10:422–27. https://doi.org/10.1016/j.arr.2011.02.001 PMID:21315186

56. Tortorella C, Pisconti A, Piazzolla G, Antonaci S. APC-dependent impairment of T cell proliferation in aging: role of CD28- and IL-12/IL-15-mediated signaling. Mech Ageing Dev. 2002; 123:1389–402. https://doi.org/10.1016/s0047-6374(02)00079-9 PMID:12297341

57. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci. 2014; 1:S4–9. https://doi.org/10.1093/gerona/glu057 PMID:24833586

58. Giunta B, Fernandez F, Nikolic WV, Obregon D, Rrapo E, Town T, Tan J. Inflammaging as a prodrome to Alzheimer's disease. J Neuroinflammation. 2008; 5:51. https://doi.org/10.1186/1742-2094-5-51 PMID:19014446

59. Frasca D, Blomberg BB. Inflammaging decreases adaptive and innate immune responses in mice and humans. Biogerontology. 2016; 17:7–19. https://doi.org/10.1007/s10522-015-9578-8 PMID:25921609

60. Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA. Age and age-related diseases: role of inflammation triggers and cytokines. Front Immunol. 2018; 9:586. https://doi.org/10.3389/fimmu.2018.00586 PMID:29686666

61. van Beek AA, Van den Bossche J, Mastroberardino PG, de Winther MP, Leenen PJ. Metabolic alterations in aging macrophages: ingredients for inflammaging? Trends Immunol. 2019; 40:113–27. https://doi.org/10.1016/j.it.2018.12.007 PMID:30626541

62. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. Nat Rev Immunol. 2014; 14:81–93. https://doi.org/10.1038/nri3600 PMID:24445666

63. Wong CK, Smith CA, Sakamoto K, Kaminski N, Koff JL, Goldstein DR. Aging impairs alveolar macrophage phagocytosis and increases influenza-induced mortality in mice. J Immunol. 2017; 199:1060–68. https://doi.org/10.4049/jimmunol.1700397 PMID:28646038

64. Shaw AC, Goldstein DR, Montgomery RR. Age-dependent dysregulation of innate immunity. Nat Rev Immunol. 2013; 13:875–87. https://doi.org/10.1038/nri3547 PMID:24157572

65. Po JL, Gardner EM, Anaraki F, Katsikis PD, Murasko DM. Age-associated decrease in virus-specific CD8+ T lymphocytes during primary influenza infection. Mech Ageing Dev. 2002; 123:1167–81. https://doi.org/10.1016/s0047-6374(02)00010-6 PMID:12044966

66. Ventura MT, Scichilone N, Paganelli R, Minciullo PL, Patella V, Bonini M, Passalacqua G, Lombardi C, Simioni L, Ridolo E, Del Giacco SR, Gangemi S, Canonica GW. Allergic diseases in the elderly: biological characteristics and main immunological and non-immunological mechanisms. Clin Mol Allergy. 2017; 15:2. https://doi.org/10.1186/s12948-017-0059-2 PMID:28174512

67. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest. 1998; 101:311–20. https://doi.org/10.1172/JCI1368 PMID:9435302

68. Yu M, Zheng X, Witschi H, Pinkerton KE. The role of interleukin-6 in pulmonary inflammation and injury induced by exposure to environmental air pollutants. Toxicol Sci. 2002; 68:488–97.

https://doi.org/10.1093/toxsci/68.2.488 PMID:12151646

69. Velazquez-Salinas L, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The role of interleukin 6 during viral infections. Front Microbiol. 2019; 10:1057. https://doi.org/10.3389/fmicb.2019.01057 PMID:31134045

70. Fehr AR, Channappanavar R, Perlman S. Middle East respiratory syndrome: emergence of a pathogenic human coronavirus. Annu Rev Med. 2017; 68:387–99. https://doi.org/10.1146/annurev-med-051215-031152 PMID:27576010

71. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

72. Bonafè M, Olivieri F, Cavallone L, Giovagnetti S, Mayegiani F, Cardelli M, Pieri C, Marra M, Antonicelli R, Lisa R, Rizzo MR, Paolisso G, Monti D, Franceschi C. A gender—dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. Eur J Immunol. 2001; 31:2357–61. https://doi.org/10.1002/1521-4141(200108)31:8<2357::aid-immu2357>3.0.co;2-x PMID:11500818

73. Franceschi C. Inflammaging as a major characteristic of old people: can it be prevented or cured? Nutr Rev. 2007; 65:S173–6. https://doi.org/10.1111/j.1753-4887.2007.tb00358.x PMID:18240544

74. Storci G, De Carolis S, Papi A, Bacalini MG, Gensous N, Marasco E, Tesei A, Fabbri F, Arienti C, Zanoni M, Sarnelli A, Santi S, Olivieri F, et al. Genomic stability, anti-inflammatory phenotype, and up-regulation of the RNAseH2 in cells from centenarians. Cell Death Differ. 2019; 26:1845–58. https://doi.org/10.1038/s41418-018-0255-8 PMID:30622304

75. Turner JM, Mead J, Wohl ME. Elasticity of human lungs in relation to age. J Appl Physiol. 1968; 25:664–71. https://doi.org/10.1152/jappl.1968.25.6.664 PMID:5727191

76. Miyazaki Y, Araki K, Vesin C, Garcia I, Kapanci Y, Whitsett JA, Piguet PF, Vassalli P. Expression of a tumor necrosis factor-alpha transgene in murine lung causes lymphocytic and fibrosing alveolitis. A mouse model of progressive pulmonary fibrosis. J Clin Invest. 1995; 96:250–59. https://doi.org/10.1172/JCI118029 PMID:7542280

77. Hinojosa E, Boyd AR, Orihuela CJ. Age-associated inflammation and toll-like receptor dysfunction prime the lungs for pneumococcal pneumonia. J Infect Dis. 2009; 200:546–54. https://doi.org/10.1086/600870 PMID:19586419

78. Tsuji T, Aoshiba K, Nagai A. Cigarette smoke induces senescence in alveolar epithelial cells. Am J Respir Cell Mol Biol. 2004; 31:643–49. https://doi.org/10.1165/rcmb.2003-0290OC PMID:15333326

79. Vellas C, Delobel P, de Souto Barreto P, Izopet J. COVID-19, virology and geroscience: a perspective. J Nutr Health Aging. 2020; 24:685–91. https://doi.org/10.1007/s12603-020-1416-2 PMID:32744561

80. Scheiblich H, Trombly M, Ramirez A, Heneka MT. Neuroimmune connections in aging and neurodegenerative diseases. Trends Immunol. 2020; 41:300–12. https://doi.org/10.1016/j.it.2020.02.002 PMID:32147113

81. Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev. 1995; 20:269–87. https://doi.org/10.1016/0165-0173(94)00015-h PMID:7550361

82. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med. 2015; 3:136. https://doi.org/10.3978/j.issn.2305-5839.2015.03.49 PMID:26207229

83. Block ML, Hong JS. Chronic microglial activation and progressive dopaminergic neurotoxicity. Biochem Soc Trans. 2007; 35:1127–32. https://doi.org/10.1042/BST0351127 PMID:17956294

84. Moseman EA, Blanchard AC, Nayak D, McGavern DB. T cell engagement of cross-presenting microglia protects the brain from a nasal virus infection. Sci Immunol. 2020; 5:eabb1817. https://doi.org/10.1126/sciimmunol.abb1817 PMID:32503876

85. Chua RL, Lukassen S, Trump S, Hennig BP, Wendisch D, Pott F, Debnath O, Thürmann L, Kurth F, Völker MT, Kazmierski J, Timmermann B, Twardziok S, et al. COVID-19 severity correlates with airway epithelium-immune cell interactions identified by single-cell analysis. Nat Biotechnol. 2020; 38:970–79. https://doi.org/10.1038/s41587-020-0602-4 PMID:32591762

86. Lampa J, Westman M, Kadetoff D, Agréus AN, Le Maître E, Gillis-Haegerstrand C, Andersson M, Khademi

M, Corr M, Christianson CA, Delaney A, Yaksh TL, Kosek E, Svensson CI. Peripheral inflammatory disease associated with centrally activated IL-1 system in humans and mice. Proc Natl Acad Sci USA. 2012; 109:12728–33.
https://doi.org/10.1073/pnas.1118748109
PMID:22802629

87. Sadasivan S, Zanin M, O'Brien K, Schultz-Cherry S, Smeyne RJ. Induction of microglia activation after infection with the non-neurotropic a/CA/04/2009 H1N1 influenza virus. PLoS One. 2015; 10:e0124047.
https://doi.org/10.1371/journal.pone.0124047
PMID:25861024

88. Jang H, Boltz D, Sturm-Ramirez K, Shepherd KR, Jiao Y, Webster R, Smeyne RJ. Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration. Proc Natl Acad Sci USA. 2009; 106:14063–68.
https://doi.org/10.1073/pnas.0900096106
PMID:19667183

89. Marreiros R, Müller-Schiffmann A, Trossbach SV, Prikulis I, Hänsch S, Weidtkamp-Peters S, Moreira AR, Sahu S, Soloviev I, Selvarajah S, Lingappa VR, Korth C. Disruption of cellular proteostasis by H1N1 influenza a virus causes α-synuclein aggregation. Proc Natl Acad Sci USA. 2020; 117:6741–51.
https://doi.org/10.1073/pnas.1906466117
PMID:32152117

90. Gagne JJ, Power MC. Anti-inflammatory drugs and risk of Parkinson disease: a meta-analysis. Neurology. 2010; 74:995–1002.
https://doi.org/10.1212/WNL.0b013e3181d5a4a3
PMID:20308684

91. Louveau A, Herz J, Alme MN, Salvador AF, Dong MQ, Viar KE, Herod SG, Knopp J, Setliff JC, Lupi AL, Da Mesquita S, Frost EL, Gaultier A, et al. CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature. Nat Neurosci. 2018; 21:1380–91.
https://doi.org/10.1038/s41593-018-0227-9
PMID:30224810

92. Da Mesquita S, Louveau A, Vaccari A, Smirnov I, Cornelison RC, Kingsmore KM, Contarino C, Onengut-Gumuscu S, Farber E, Raper D, Viar KE, Powell RD, Baker W, et al. Functional aspects of meningeal lymphatics in ageing and Alzheimer's disease. Nature. 2018; 560:185–191.
https://doi.org/10.1038/s41586-018-0368-8
PMID:30046111

93. Bostancıklıoğlu M. SARS-CoV2 entry and spread in the lymphatic drainage system of the brain. Brain Behav Immun. 2020; 87:122–23.
https://doi.org/10.1016/j.bbi.2020.04.080
PMID:32360606

94. Ye M, Ren Y, Lv T. Encephalitis as a clinical manifestation of COVID-19. Brain Behav Immun. 2020; 88:945–46.
https://doi.org/10.1016/j.bbi.2020.04.017
PMID:32283294

95. Paniz-Mondolfi A, Bryce C, Grimes Z, Gordon RE, Reidy J, Lednicky J, Sordillo EM, Fowkes M. Central nervous system involvement by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). J Med Virol. 2020; 92:699–702.
https://doi.org/10.1002/jmv.25915
PMID:32314810

96. Varatharaj A, Thomas N, Ellul MA, Davies NW, Pollak TA, Tenorio EL, Sultan M, Easton A, Breen G, Zandi M, Coles JP, Manji H, Al-Shahi Salman R, et al, and CoroNerve Study Group. Neurological and neuropsychiatric complications of COVID-19 in 153 patients: a UK-wide surveillance study. Lancet Psychiatry. 2020; S2215-0366:30287.
https://doi.org/10.1016/S2215-0366(20)30287-X
PMID:32593341

97. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, Chang J, Hong C, Zhou Y, Wang D, Miao X, Li Y, Hu B. Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. JAMA Neurol. 2020; 77:1–9.
https://doi.org/10.1001/jamaneurol.2020.1127
PMID:32275288

98. Ellul MA, Benjamin L, Singh B, Lant S, Michael BD, Easton A, Kneen R, Defres S, Sejvar J, Solomon T. Neurological associations of COVID-19. Lancet Neurol. 2020; 19:767–83.
https://doi.org/10.1016/S1474-4422(20)30221-0
PMID:32622375

99. Lippi A, Domingues R, Setz C, Outeiro TF, Krisko A. SARS-CoV-2: at the crossroad between aging and neurodegeneration. Mov Disord. 2020; 35:716–20.
https://doi.org/10.1002/mds.28084
PMID:32291797

100. Gardner A, Autin L, Barbaro B, Olson AJ, Goodsell DS. CellPAINT: interactive illustration of dynamic mesoscale cellular environments. IEEE Comput Graph Appl. 2018; 38:51–66.
https://doi.org/10.1109/MCG.2018.2877076
PMID:30668455

Research Paper

Shorter telomere lengths in patients with severe COVID-19 disease

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ABSTRACT

The incidence of severe manifestations of COVID-19 increases with age with older patients showing the highest mortality, suggesting that molecular pathways underlying aging contribute to the severity of COVID-19. One mechanism of aging is the progressive shortening of telomeres, which are protective structures at chromosome ends. Critically short telomeres impair the regenerative capacity of tissues and trigger loss of tissue homeostasis and disease. The SARS-CoV-2 virus infects many different cell types, forcing cell turn-over and regeneration to maintain tissue homeostasis. We hypothesize that presence of short telomeres in older patients limits the tissue response to SARS-CoV-2 infection. We measure telomere length in peripheral blood lymphocytes COVID-19 patients with ages between 29 and 85 years-old. We find that shorter telomeres are associated to increased severity of the disease. Individuals within the lower percentiles of telomere length and higher percentiles of short telomeres have higher risk of developing severe COVID-19 pathologies.

INTRODUCTION

The current COVID-19 pandemic (https://www.who.int/) is produced by the SARS-CoV-2 virus, a novel zoonotic Coronavirus of the betacoronavirus genus that most likely crossed species from bats to humans leading to a pneumonia outbreak initially reported in Wuhan, China and now affecting the majority of countries. SARS-CoV-2 causes from mild flu-like symptoms in approximately 80% of the cases to a severe lung and multi-organic failure which can result in death of a significant percentage of patients. Pathologies associated with SARS-CoV-2 include severe lung failure, diarrhea, heart infarct, and brain pathologies among others [1–3]. This wide viral tropism is mediated by expression of the Angiotensin-converting enzyme 2 (ACE2), which acts as the receptor protein for the virus to enter the host cells. In particular, the SARS-CoV-2 spike protein directly binds the ACE2 human protein [4–7]. The human ACE protein is expressed in alveolar type II (ATII) cells in the lung [8], as well as in the kidney, the heart and the gut [9–14]. This expression pattern of the ACE protein explains that a preferential site for SARS-CoV-2 infection is the lung [4, 15, 16], although the virus can also infect kidney, intestine, and heart cells causing severe pathologies in all these tissues [1–3, 11, 17, 18]. In this regard, it caught our attention that a common outcome of SARS-CoV-2 infection seems to be induction of fibrosis-like phenotypes in the lung and kidney, suggesting that the viral infection maybe exhausting the regenerative potential of tissues [11, 16–18].

In contrast to influenza infection, that causes a high mortality in infants [19–24], SARS-CoV-2 infection causes low mortality in infants or children but results in a progressively increased mortality with increasing age reaching up to 15% mortality in individuals that are ≥80 years old (see https://covid19.isciii.es/ for mortality data in Spain). These findings suggest that molecular mechanisms at the origin of organismal aging maybe

influencing the outcome of SARS-CoV-2 infection by increasing lethality. One of such molecular events underlying aging is the progressive shortening of telomeres throughout life, which can cause exhaustion of the proliferative potential of stem cells and immune cells among others [25–27].

Telomeres are specialized structures at the chromosome ends, which are essential for chromosome-end protection and genomic stability [28]. Vertebrate telomeres consist of tandem repeats of the TTAGGG DNA sequence bound by a six-protein complex known as shelterin, which prevents chromosome end-to-end fusions and telomere fragility [29, 30]. As cells divide and DNA has to be replicated, telomeres become progressively shorter owing to the so-called "end replication problem" [31, 32]. Thus, telomere shortening occurs associated with increasing age in humans [33], mice [34] and other species, and the rate of telomere shortening has been shown to correlate with species lifespan [35]. When telomeres become critically short this results in loss of telomere protection, leading to activation of a persistent DNA damage response [36] and loss of cellular viability by induction of apoptosis and/or senescence [30].

Telomerase is a reverse transcriptase that is able to elongate telomeres *de novo* by adding TTAGGG repeats to chromosome ends [37]. Telomerase is active in embryonic stem cells, thereby ensuring sufficiently long telomeres with generations in a given species. After birth, however, telomerase expression is silenced in the majority of cell types causing telomeres to shorten with age.

We have shown by using telomerase-deficient mice with critically short telomeres, that short telomeres are sufficient to impair the ability of stem cells to regenerate different tissues, including skin, brain and bone marrow [38–41]. In humans, mutations in telomerase or telomere-binding proteins can also lead to very short telomeres and appearance of pathologies characterized by loss of the regenerative capacity of tissues and presence of fibrosis in lungs, liver or kidney, as well as by intestinal atrophy and bone marrow aplasia [42].

In particular, we previously demonstrated that short or dysfunctional telomeres are at the origin of pulmonary fibrosis in mouse models of the disease [43]. In particular, induction of telomere dysfunction specifically in alveolar type II (ATII) cells by deletion of an essential telomere protective protein in these cells, TRF1, is sufficient to induce progressive and lethal pulmonary fibrosis phenotypes in mice, which are concomitant with induction of telomeric DNA damage, cell death and senescence [43]. These findings demonstrate that dysfunctional telomeres in lungs ATII cells lead to loss of viability of these cells and induction of fibrosis. Also in support of this notion, we have demonstrated that therapies aimed to elongate telomeres, such as a telomerase gene therapy using adeno-associated vectors (AAV9-TERT) can stop the progression of pulmonary fibrosis associated to short telomeres in mouse models of the disease by increasing telomere length in ATII cells, as well as their proliferative potential [44], thus demonstrating the importance of sufficiently long telomeres to allow tissue regeneration.

Importantly, as SARS-CoV-2 infects different cell types in humans, including ATII cells in the lungs, it is plausible that viral infection could damage these different cell types forcing an increased turn-over of different regenerative cell types. While in young individuals with sufficiently long telomeres, regenerative cell types, such as lung ATII cells could undergo these extra cell divisions and contribute to tissue healing, older individuals with shorter telomeres may fail to allow cell proliferation and regeneration, thus leading to tissue failure. Thus, here we set to assess whether telomere length in COVID-19 patients correlated with development of more severe COVID-19 pathologies.

RESULTS

Pathologies in COVID-19 patient cohort

In order to assess the potential impact of telomere length on pathologies associated to COVID-19 disease, we obtained both DNA and mononuclear cells from peripheral blood samples from patients hospitalized at the IFEMA field hospital in Madrid, which was constructed to treat COVID-19 patients. A total of 61 female and 28 male patients of ages ranging from 29 to 85 years old were included in the study (Table 1). The patient cohort had different severity of pathologies and received the treatments indicated in Table 1. None of the patients included in this study died as a consequence of the COVID-19 disease.

In order to correlate patient severity with telomere length, we first grouped the patients according the a severity score ranging from 1 to 4, with severity score 1 in the case of patients with low fever and cough but without any radiological features of pneumonia to patients with severity score of 4 in the cases of patients with features of Acute Respiratory Distress Syndrome (ARDS) requiring mechanical ventilation along with presence of multiorgan dysfunction failure, metabolic acidosis and coagulation dysfunction (Materials and Methods).

Table 1. Patients included in this study.

Age	Sex	COVID-19 severity	Treatment
29	Male	Moderate	Hydroxychloroquine, Azitromicine
30	Female	Moderate	Dolquine, Azitromicine
31	Female	Moderate	Hydroxychloroquine, Azitromicine, Kaletra
33	Female	Moderate	Hydroxychloroquine, Azitromicine
33	Female	Severe	Hydroxychloroquine, Azitromicine
35	Male	Moderate	Hydroxychloroquine, Ceftriaxone, Azitromicine, Kaletra
36	Female	Acute	Hydroxychloroquine, Kaletra, Corticoids,Ceftriaxone, Azitromicine, LINEZOLID
38	Female	Mild	Hydroxychloroquine
39	Female	Moderate	Hydroxychloroquine, Azitromicine
40	Male	Severe	Hydroxychloroquine, Azitromicine,Ceftriaxone, systemic corticoids
41	Female	Moderate	Hydroxychloroquine, Kaletra
42	Male	Severe	Hydroxychloroquine, Kaletra, Corticoids
43	Female	Mild	Hydroxychloroquine, Azitromicine
43	Female	Severe	Hydroxychloroquine, Azitromicine, Corticoids, Kaletra, Tocilizumab
43	Female	Moderate	Hydroxychloroquine, Azitromicine
44	Male	Mild	Hydroxychloroquine
45	Female	Moderate	Dolquine, Kaletra, Azitromicine
45	Female	Severe	Kaletra, Hydroxychloroquine, Azitromicine, Corticoids
46	Female	Moderate	Kaletra, Dolquine, Colchicine
46	Female	Severe	Dolquine, Kaletra, Azitromicine, Corticoids
46	Female	Severe	Hydroxychloroquine, Azitromicine
47	Female	Moderate	Hydroxychloroquine
47	Male	Severe	Hydroxychloroquine, Azitromicine, systemic corticoids
47	Female	Mild	Hydroxychloroquine,Ceftriaxone
48	Male	Severe	Hydroxychloroquine, Azitromicine, Tocilizumab, Kaletra
49	Female	Moderate	Hydroxychloroquine, Azitromicine
49	Male	Severe	Hydroxychloroquine, Azitromicine, systemic corticoids, Tocilizumab
49	Male	Moderate	Hydroxychloroquine, Azitromicine
49	Male	Moderate	Hydroxychloroquine, Azitromicine,Ceftriaxone
49	Male	Moderate	Hydroxychloroquine, Kaletra
49	Female	Moderate	Hydroxychloroquine, Azitromicine
50	Female	Moderate	Hydroxychloroquine, Azitromicine
51	Male	Severe	Hydroxychloroquine, Azitromicine
51	Female	Moderate	Hydroxychloroquine, Azitromicine, Kaletra,Ceftriaxone
51	Female	Moderate	Hydroxychloroquine, Kaletra, Azitromicine, Corticoids
52	Female	Severe	Chloroquine, Kaletra, Tocilizumab, methylprednisolone
52	Male	Severe	Hydroxychloroquine
52	Female	Moderate	Hydroxychloroquine, Azitromicine
53	Female	Severe	Hydroxychloroquine, Corticoids, Tocilizumab
53	Male	Moderate	Hydroxychloroquine, Azitromicine
53	Male	Severe	Hydroxychloroquine, Azitromicine, Cortocoid therapy
54	Female	Acute	Hydroxychloroquine, Kaletra, Azitromicine, INTERFERON, Tocilizumab, Corticoids
54	Female	Acute	Hydroxychloroquine, Azitromicine, systemic corticoids, Tocilizumab
54	Female	Severe	Hydroxychloroquine, Azitromicine, Kaletra, systemic corticoids
54	Female	Severe	Hydroxychloroquine, Azitromicine, Corticoids, Tocilizumab
54	Female	Moderate	Hydroxychloroquine
54	Female	Moderate	Hydroxychloroquine, Azitromicine,Ceftriaxone

54	Female	Moderate	Hydroxychloroquine, Kaletra, Azitromicine
55	Female	Mild	Hydroxychloroquine
55	Female	Mild	Ceftriaxone, Hydroxychloroquine
55	Male	Moderate	Hydroxychloroquine
56	Female	Moderate	Dolquine, Azitromicine
56	Female	Severe	Hydroxychloroquine, Azitromicine, systemic corticoids
56	Male	Moderate	Hydroxychloroquine, Azitromicine
56	Female	Severe	REMDESIVIR, Corticoids, Tocilizumab
57	Male	Severe	Chloroquine, Corticoids, Interferon beta
57	Male	Acute	Hydroxychloroquine, Azitromicine, Corticoids, Tocilizumab
57	Female	Severe	Hydroxychloroquine, Kaletra, Corticoids
57	Male	Moderate	Hydroxychloroquine, Kaletra
57	Female	Severe	Hydroxychloroquine, Corticoids,Tocilizumab, Azitromicine
58	Male	Severe	Hydroxychloroquine, Azitromicine, systemic corticoids
58	Female	Moderate	Hydroxychloroquine+Azitromicine
58	Female	Severe	Hydroxychloroquine, Azitromicine, Methylprednisone
59	Female	Severe	Hydroxychloroquine, Azitromicine, Corticoids
59	Female	Moderate	Hydroxychloroquine, Kaletra, Predisolone
60	Female	Severe	Hydroxychloroquine, Azitromicine, Corticoids, Tocilizumab
60	Female	Moderate	Hydroxychloroquine+Azitromicine+Kaletra
61	Female	Moderate	Dolquine, Azitromicine
61	Male	Severe	Hydroxychloroquine, Ceftriaxone, Azitromicine, systemic corticoids
62	Female	Moderate	Hydroxychloroquine, Azitromicine
62	Female	Severe	Hydroxychloroquine, Azitromicine, Kaletra
63	Female	Severe	Hydroxychloroquine, Azitromicine, Dexamethasone
63	Male	Severe	Hydroxychloroquine, Azitromicine, Corticoids
65	Male	Severe	Hydroxychloroquine, Kaletra, Corticoids
65	Male	Severe	Hydroxychloroquine, Azitromicine, Kaletra, Dexamethasone, Tocilizumab
66	Female	Severe	Hydroxychloroquine, Kaletra, systemic corticoids
67	Female	Moderate	Hydroxychloroquine
67	Female	Severe	Chloroquine, Kaletra, Azitromicine, Tocilizumab, Corticoids
67	Male	Severe	Hydroxychloroquine, Azitromicine, Corticoids, Kaletra
69	Male		
70	Female	Severe	Dolquine, Kaletra, Azitromicine, Corticoids
71	Male	Severe	Hydroxychloroquine, Kaletra, Corticoids
71	Female	Severe	Hydroxychloroquine, Azitromicine, Cortocoid therapy
72	Female	Acute	Hydroxychloroquine, Azitromicine,systemic corticoids
73	Female	Severe	Hydroxychloroquine, Azitromicine, Kaletra, systemic corticoids, Tocilizumab
74	Female	Severe	Hydroxychloroquine, Azitromicine, Corticoids
77	Female	Severe	Hydroxychloroquine
81	Female	Mild	Hydroxychloroquine, Azitromicine,Ceftriaxone
85	Female	Moderate	Hydroxychloroquine, Kaletra, Azitromicine

Determination of telomere length in COVID-19 patients

In order to determine telomere length in our patient cohort, peripheral blood was extracted from the arm from the different patients and used to measure telomere length by three independent techniques (Materials and Methods). First, we determined telomere length in DNA extracted from peripheral blood by using both the Southern blotting-based Telomere Restriction Analysis (TRF; see [38]) and the quantitative-PCR (qPCR) telomere length analyses [45]. In addition to these two technologies based on DNA samples, we also measured telomere length on fresh peripheral blood mononuclear cells by using the

more precise high- throughput quantitative fluorescence *in situ* hybridization (HT Q-FISH) previously described by us [34, 35, 46], which allows determination of individual telomere fluorescence signals using tens of thousands of cells from a single patient. The fact that HT Q-FISH can determine individual telomere fluorescence spots in interphasic nuclei, each spot usually formed by several clustered telomeres, allows to the determine the abundance of very short telomeres.

We observed a very significant correlation between the telomere length measurements obtained by the three different techniques (Figure 1A), thus indicating the robustness of the data on telomere length obtained in our patient cohort. Given the good correlation of the telomere length data obtained with the three technologies, we decided to perform the rest of the analysis with the telomere length data obtained by HT Q-FISH, as it measures telomeres in a single cell manner and it also allows to measure individual telomere spots within single nuclei.

The rate of telomere shortening in the patient cohort was of 77 bp/year (Figure 1B). This rate of telomere shortening is in the range previously published by us and others [45–48]. In agreement with telomere shortening with increasing patient age, we also observed an increase in the abundance of short telomeres

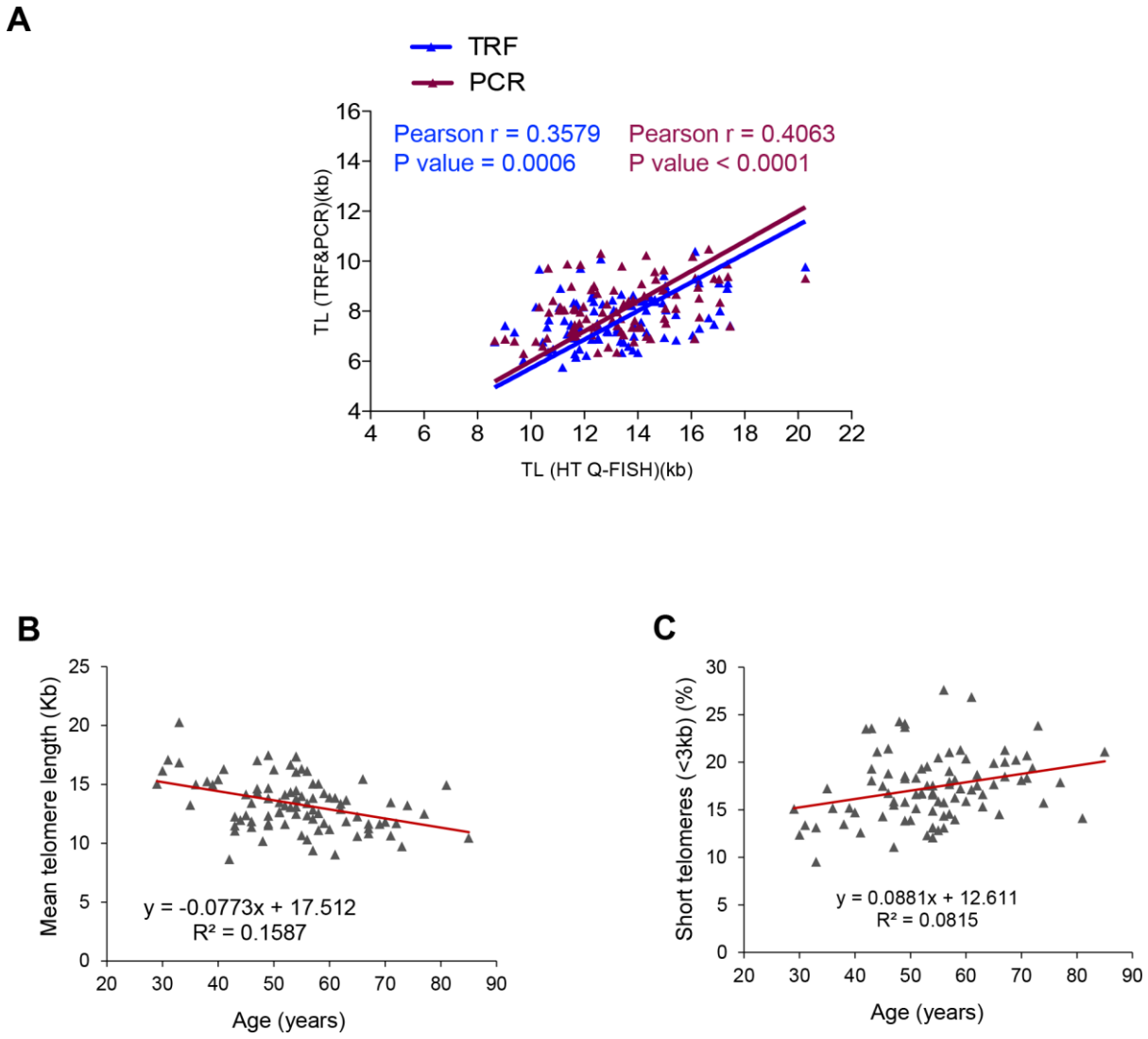

Figure 1. Correlation between HT Q-FISH and PCR and TRF techniques for telomere length measurements. (**A**) Correlation of telomere length measured by TRF, qPCR and HT Q-FISH in Peripheral blood mononuclear cell (PMBC) samples from 89 individuals. (**B**, **C**) Linear regression analysis was used to determine the rate of telomere shortening (**B**) and the rate of the increase of short telomeres (<3kb) (**C**) per year in PMBCs. The telomere length data in (**B**, **C**) correspond to HT-qFish analysis. The Pearson correlation coefficient and linear regression equation were determined using GraphPad Software.

(ie, telomere fluorescence spots corresponding to less than 3 kb of telomere length) which increased at a rate of 8.8 % per year (Figure 1B).

When we analyzed the data segregated by gender, linear regression of telomere length data in COVID-19 female patients showed that their telomeres were consistently longer than those of male patient at all age ranges, as well as they showed a lower percentage of short telomeres compared to male patients (Figure 2A–2D), also in agreement with previous findings [46]. Again, when segregated by gender, the rates of telomere shortening were in a range of 70-80 bp/year (Figure 2A). Similarly, the increase in the percentage of short telomeres with age was also similar in both genders (Figure 2A, 2B).

The fact that COVID-19 female patients had longer telomeres than men patients at different age ranges is in line with the fact that female COVID-19 patients show a lower mortality than males (see COVID-19 Sex-Disaggregated Data Tracker available at: http://globalhealth5050.org/covid19).

Age and telomere length correlate with COVID-19 severity

In order to assess whether short telomeres correlated with the severity of COVID-19 disease, we used a Pearson correlation analysis between the mean telomere length or the percentage of short telomeres (<3 kb) as determined by the HT Q-FISH technique, and either age or the severity score of the different COVID-19 patients ranging from 1 (less severe) to 4 (more severe) (see Materials and Methods).

As expected, we observed a significant inverse correlation between mean telomere length (TL) and age of the COVID-19 patients (r=-0.3985; p=0.0001; Figure 3A). We also observed a significant direct correlation between percentage of short telomeres (ie, telomeres < 3Kb) and patient age (r=0.285; p=0.0067; Figure 3B). Thus, these findings confirm a significantly higher incidence of short telomeres with increasing age in the COVID-19 patients. We also observed an inverse correlation between mean telomere length (TL) and the severity score of the COVID-19 patients when using HT Q-FISH (r=-0.1752; p=0.1026; Figure 3A) and a direct correlation between the percentage of short telomeres (ie, telomeres < 3Kb) and the severity score (r=0.1454; p=0.1766; Figure 3B), although these correlations did not reach statistical significance. To further analyze this, we performed similar analysis with the telomere length data obtained by TRF and by PCR (Figure 3C, 3D). Again, we confirmed a significant inverse correlation between mean telomere length (TL) and age of the COVID-19 patients by TRF (r=-0.4675; p<0.0001; Figure 3C) as well as by PCR (r=-0.405; p=0.0001; Figure 3D) techniques. Importantly, by these two DNA-based techniques, the correlation between telomere length and COVID-19 severity reached statistical significance (TRF: r=-0.3119, p=0.004; Figure 3C; PCR: r= -0.2308, p=0.036; Figure 3D.

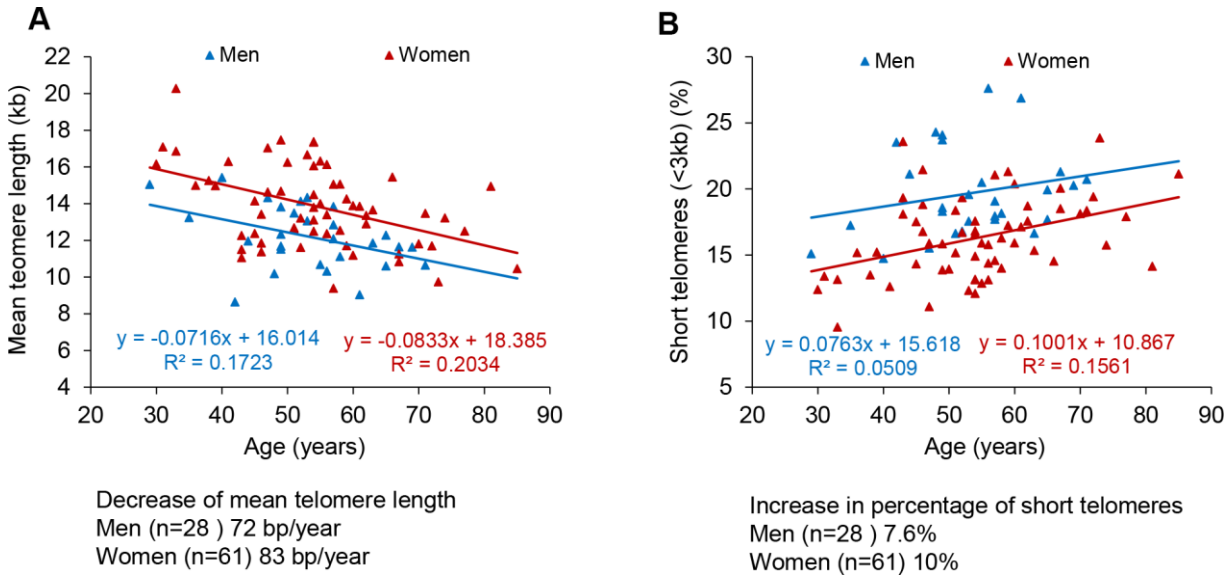

Figure 2. Telomere shortening and of increase in short telomeres with age in men and women. (**A**, **B**) Percentage of short telomeres (<3 kb) in PMBC samples. Mean telomere length (**A**) and percent of short telomeres (<3kb) (**B**) in PMBCs from male (blue) and female (red) patients. Linear regression analysis was used to assess the rate of telomere shortening expressed as number of bp loss and the increase of the percentage of short telomeres per year.

Finally, we also observed a significant direct correlation between age of the COVID-19 patients and the severity score of the disease (r=0.2299; p=0.0312; Figure 4A, 4B). Furthermore, we observed an inverse correlation between age and mean telomere length (TL) and a direct correlation between age and the percentage of short telomeres when using HT Q-FISH (Figure 4A, 4B).

Together, these findings suggest significant correlations of age as well as telomere length with COVID-19 severity.

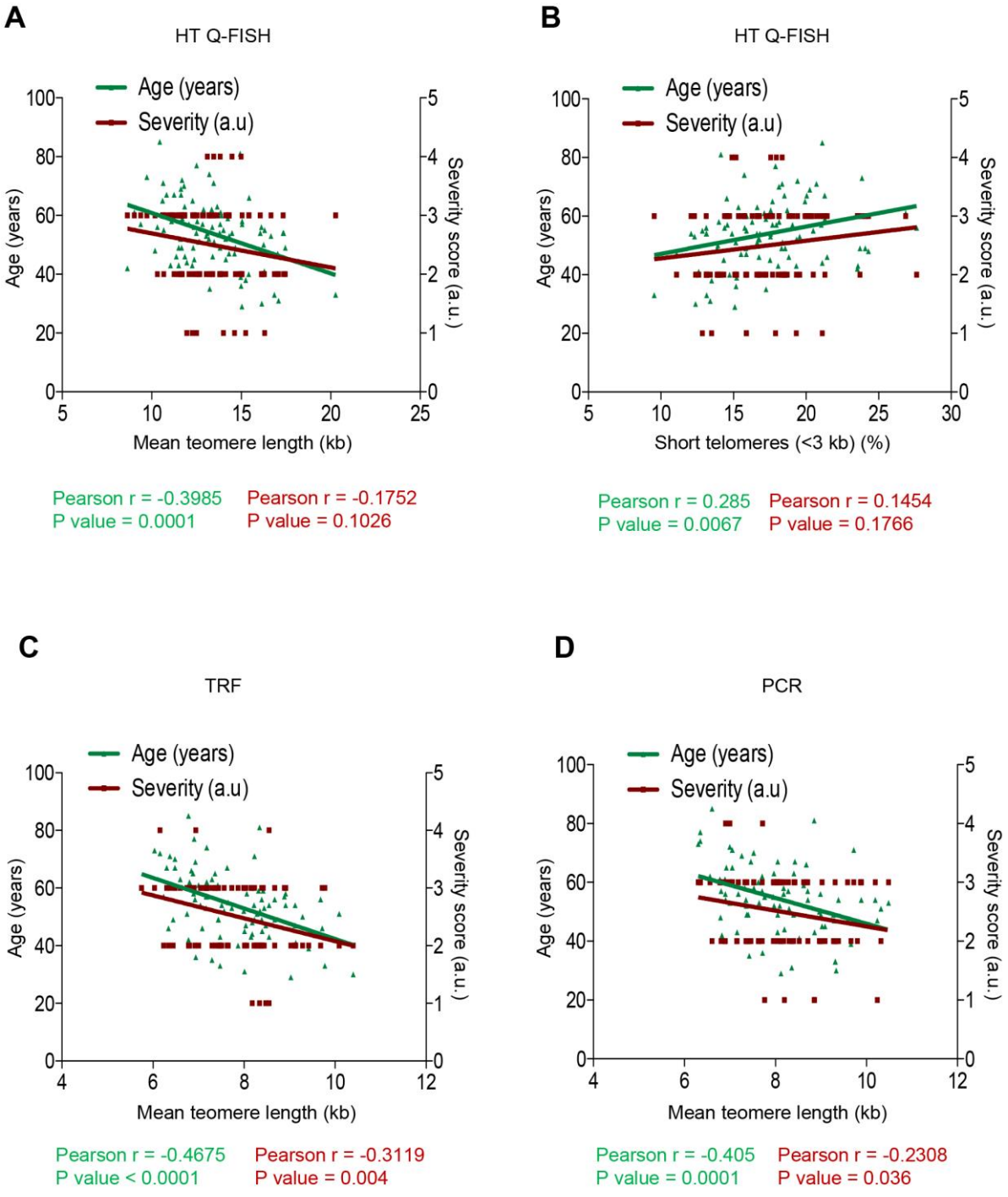

Figure 3. Correlation between telomere length, age and COVID-19 severity. (**A**–**D**) Pearson correlation analysis between telomere length (**A**, **C**, **D**) or percentage of short telomeres (<3 kb) (**B**) and age or COVID-19 severity in PMBC samples. In (**A**, **B**) telomere length was analysed by HT-QFISH and in (**C**, **D**) by TRF and PCR, respectively. The severity score was established by assigning values of 1, 2, 3, 4 for mild, moderate, severe, and acute, respectively (see Materials and Methods). The Pearson r coefficient and the P values are indicated.

Higher severity of COVID-19 disease in patients in the lower percentiles of telomere length

The findings suggest that COVID-19 patients with shorter telomere length may have a higher risk of more severe pathologies. To further test this, we divided the patients in quartiles according to either their mean telomere length or their percentage of short telomeres using the telomere signal fluorescence data obtained by HT Q-FISH. We observed that patients in the lower quartile of mean telomere length (<25%) had a higher severity score (p=0.06; Figure 5A). Similarly, the

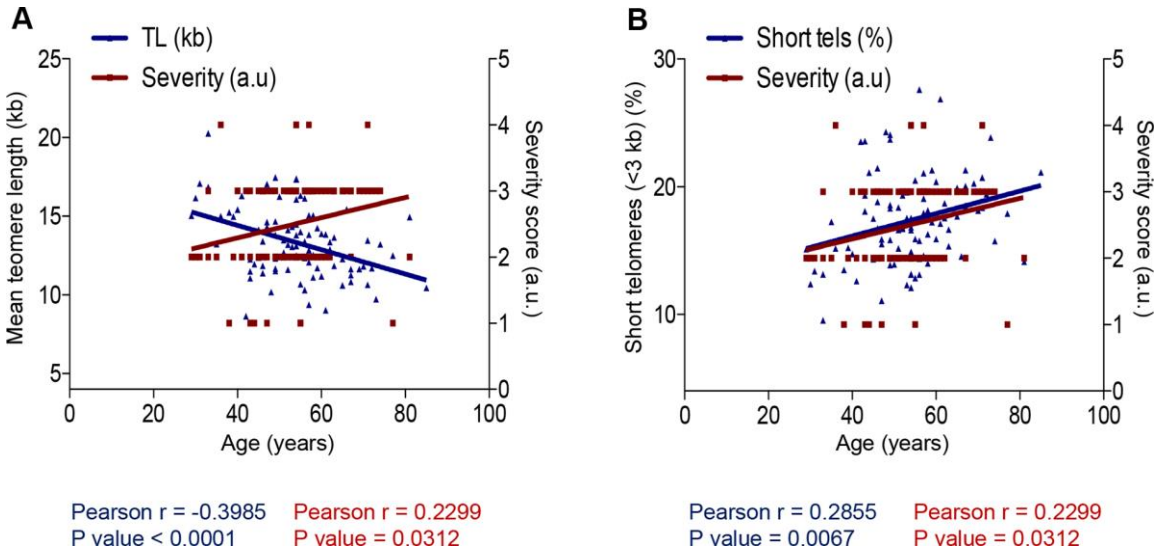

Figure 4. Correlation between age and COVID-19 severity and telomere length. (**A**, **B**) Person correlation analysis between age and telomere length measured by HT Q-FISH in PMBC samples (**A**) and with percentage of short telomeres (<3 kb). The severity score was established by assigning values of 1, 2, 3, 4 for mild, moderate, severe and acute, respectively (see Materials and Methods). The Pearson r coefficient and the P values are indicated.

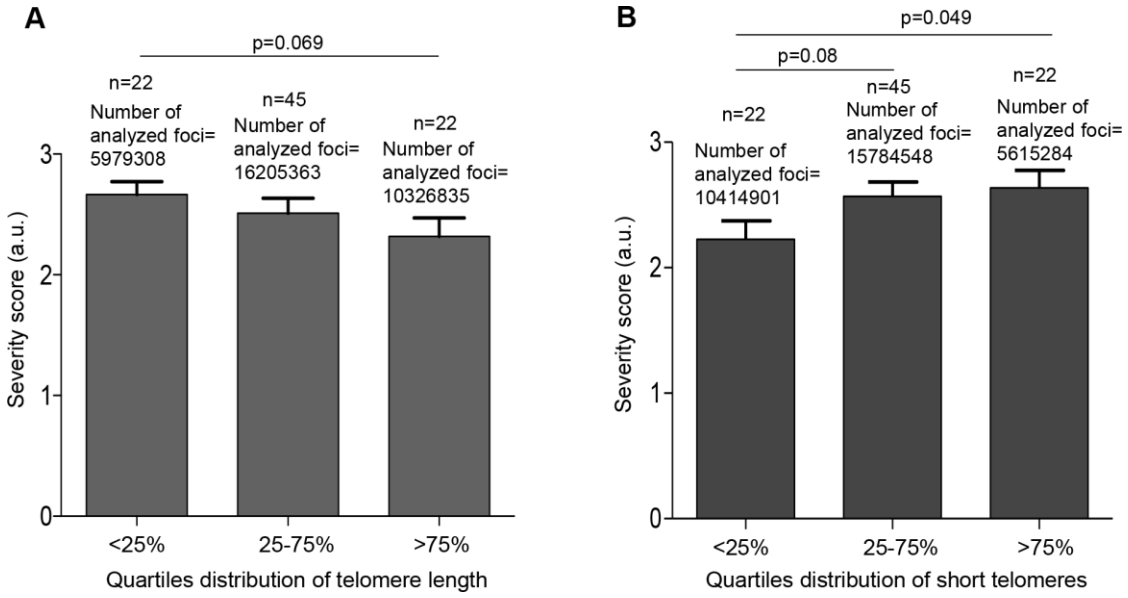

Figure 5. Patients with shorter telomeres develop more severe COVID-19 disease. (**A**) The telomere lengths of patients were distributed into the quartiles <25% (<11.68 kb), 25-75% (11.68–14.96 kb) and >75% (>14.96 kb) and correlated with COVID-19 severity. (**B**) The abundance of short telomeres was distributed into the quartiles <25% (<14.73%), 25-75% (14.73-19.32%) and >75% (>19.32%) and correlated with COVID-19 severity. Data represent mean values ±SEM. Statistical significance was assessed using Student's t test.

patients in the higher quartile of percentage of short telomeres had significantly higher severity scores of the disease (p=0.049; Figure 5B).

Different rates of telomere shortening in patients with different severity scores

As patients in the lower quartile of telomere length have a significantly higher risk of severe COVID-19 pathologies, we set to investigate whether the rates of telomere shortening in higher severity score patients were significantly higher than in the lower severity score patients. To this end, we pooled together the patients in "mild-moderate" and "severe-acute" severity groups. We found that patients with a "severe-acute" diagnosis showed a significantly faster rate of telomere shortening compared to the "mild-moderate" diagnosis as determined by HT Q-FISH (p=0.024; Figure 6A). Of note, patients with "severe-acute" COVID-19 disease have shorter telomeres along all age groups compared to patients with "mild-moderate" COVID-19 disease. Similarly, we found an increased rate of accumulation

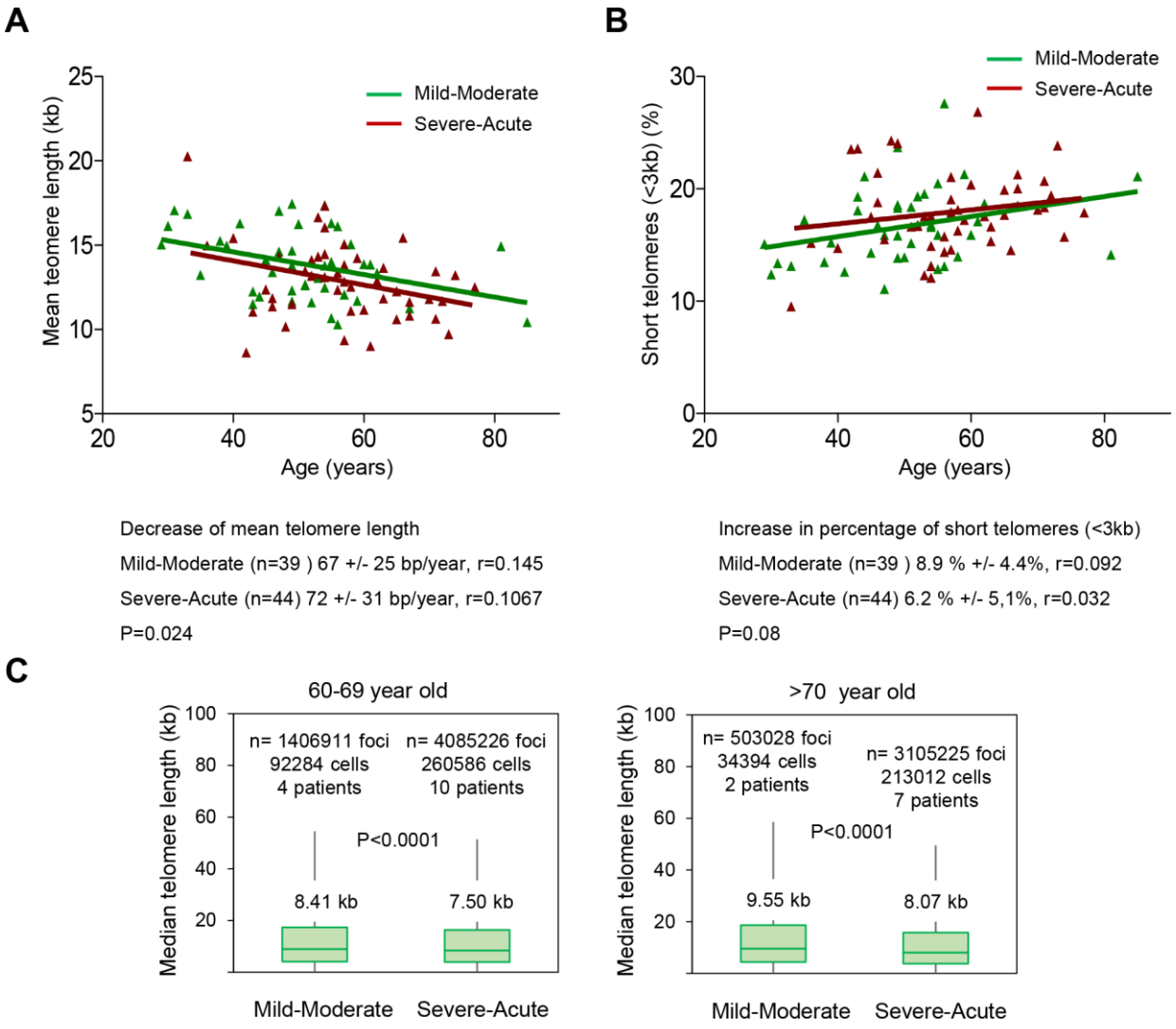

Figure 6. Patients with a higher COVID-19 severity score show faster telomere shortening rates. (**A**, **B**) Telomere shortening (**A**) and increase in percent of short telomeres (<3kb) (**B**) with age in patients diagnosed with mild-moderate and severe-acute COVID-19. Linear regression analysis was used to assess the number of bp loss and of the percent of short telomeres per year (<3 kb) in PMBC of these donors. Statistical significance was assessed using the Mann-Whitney test. (**C**) Whisker plot representation of telomere length. The between 60-69 and older than 70-year-old were pooled together within the same age group. The patients diagnosed with mild or moderate and those diagnosed with severe or acute were grouped. The telomere length corresponding to individual telomere foci were plotted according to Covid-19 severity groups. The ends of the box are the upper and lower quartiles so that the box spans the interquartile range. The middle line represents the median and bars to standard deviation. The statistical significance was calculated by one way Anova with post tukey test. n= number of foci.

of short telomeres in patients with a "severe-acute" diagnosis compared to patients with a "mild-moderate" severity score (p=0.08; Figure 6B).

In order to address whether this association between Covid-19 severity and short telomeres was independent of the age, we grouped the patients in different age groups (below 40 years of age; between 40-49; 50-59; 60-69 and over 70 years of age) and compared the fluorescence of individual telomere foci in patients showing either "mild-moderate" or "severe-acute" COVID-19 severity. We found that for age groups above 60 years of age, telomeres were shorter in the groups with "severe-acute" severity compared to "mild-moderate" severity (Figure 6C).

DISCUSSION

Data from COVID-19 around the world shows that patients of older age groups show a higher severity of the disease and a higher mortality. Male patients also show a higher mortality than female patients (see COVID-19 Sex-Disaggregated Data Tracker available at: http://globalhealth5050.org/covid19). This suggest that molecular mechanisms of aging maybe aggravating the pathological consequences of infection by the SARS-CoV-2 virus. Telomere shortening and accumulation of DNA damage steaming from short telomeres has been proposed as one of the primary hallmarks of aging [27]. In particular, short telomeres are known to result in chromosomal instability and loss of cell viability by inducing replicative senescence and/or apoptosis [26]. Importantly, by using mouse models that lack telomerase activity, we and others have shown that short telomeres impair the regenerative capacity of tissues leading to loss of tissue homeostasis and degenerative diseases [40]. Similarly, humans with critically short telomeres owing to mutations in telomerase also show an impaired regeneration capacity and are at a higher risk of developing degenerative diseases in both low proliferative (lung, kidney) and high proliferative tissues (bone marrow, skin) [42].

Given that the SARS-CoV-2 virus infects different cell types in the organisms, including regenerative cell types such as alveolar type II (ATII) cells in the lungs [8–13, 49], here we hypothesize that individuals with short telomeres would have an impaired regenerative response upon SARS-CoV-2 infection, thus leading to more severe and progressive pathologies, such as fibrosis-like pathologies in the lungs, kidney or liver.

To address this, we have measured telomere length in a total of 89 patients diagnosed with COVID-19 ranging from mild to acute disease. As expected we found that telomere length decreases with age, with women having longer telomeres than men at different age groups, which could explain why COVID-19 disease is more severe in males than females. Interestingly, we also found that those patients that have more severe COVID-19 pathologies have shorter telomeres at different ages compared to the patients with milder disease. Indeed, patients which are in the lower percentile of telomere length also have significantly higher severity scores.

These findings demonstrate that molecular hallmarks of aging, such as presence of short telomeres can influence the severity of COVID-19 pathologies. As short telomeres can be elongated by telomerase, and telomerase activation strategies have been shown by us to delay aging and age related pathologies [50], as well as to have therapeutic effects in diseases associated to short telomeres, such as pulmonary fibrosis [44], it is tempting to speculate that such telomerase activation therapies could ameliorate some of the tissue pathologies remaining in COVID-19 patients, such as fibrosis-like pathologies in the lungs [51] after overcoming the viral infection.

MATERIALS AND METHODS

Patients

In this study participated a total of 89 patients (61 female and 28 male patients of ages ranging from 29 to 85 years old) from the IFEMA field hospital installed due to the emergency situation in Madrid, Spain. All these samples were donated to CNIO BioBank which allows their use for biomedical analyses under the existing law requirements in Spain.

Blood samples

A total of 8 ml of blood were collected from the arm of each patient in heparin tubes and 4 ml in EDTA tubes and shipped within less than 24h to the DNA National Bank at Salamanca University, where they were immediately processed at a biosafety level (BSL) 3 (BSL-3) biocontainment level. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll gradient and frozen in 90% FBS (v/v) supplemented with 10% (v/v) DMSO into a number of aliquots ranging from 1 to 3, according to cell number. PBMCs were stored in vapor phase-nitrogen.

Genomic DNA was extracted directly from blood samples using the Real Blood DNA Kit and stored long-term in TE at -20° C.

Q-PCR Assay to measure average telomere length

Telomere length was measured in genomic DNA isolated from blood samples. We used a modified

monochrome multiplex quantitative polymerase chain reaction (PCR) method already described [45]. Briefly, each reaction included SYBR Green I (Promega), telomere primer pair telg (5′-ACACTAAGGTTTGG GTTTGGGTTTGGGTTTGGGTTAGTGT-3′) and telc (5′-TGTTAGGTATCCCTATCCCTATCCCTATCCCT ATCCCTAACA-3′) (final concentrations 900nM each), albumin primer pair albu (5′-CGGCGGCGGGCGGCG CGGGCTGGGCGGaaatgctgcacagaatccttg-3′) and albd (5′-GCCCGGCCCGCCGCGCCCGTCCCGCCGgaaaag catggtcgcctgtt-3′) (final concentrations 900nM each) and 20 ng of genomic DNA. The Applied Biosystems QuantStudio 6 Flex Real-Time PCR System was used. The thermal cycling profile was Stage 1: 15 min at 95° C; Stage 2: 2 cycles of 15 s at 94° C, 15 s at 49° C; and Stage 3: 32 cycles of 15 s at 94° C, 10 s at 62° C, 15 s at 74° C with signal acquisition, 10 s at 84° C, 15 s at 88° C with signal acquisition. To serve as a reference for standard curve calculation, HeLa cells were serially diluted and qPCR performed as described above. After thermal cycling was completed, the QuantStudio 6 software was used to generate standard curves and Ct values for telomere signals and reference gene signals. The average telomere length was termed T/S ratio. Finally, T/S ratios were converted into kb by external calibration with the K562 (6.5 kb), CCRF-CEM (7.5 kb), Jurkat (11.5 kb) and HeLa1211 (24 kb) cell lines.

Terminal restriction fragment analysis

Mean telomere length by Telomere Restriction Fragment (TRF) was determined using the method already described [38]. Briefly, genomic DNA was digested by MboI and separated by gel electrophoresis in 0.5X TBE maintained at 14° C, using a CHEF DR-II pulsed-field apparatus (BioRad) for 14 h at 5 V/cm at a constant pulse time of 0.5 s. The gel was transferred to a nylon membrane (Hybond-XL, GE Healthcare) and probed with a ^{32}P-labeled telomeric probe (TTAGGG)n (a gift from T. de Lange). Mean TRF lengths were determined using an ImageQuant TL.

HT Q-FISH

Clear bottom black-walled 96-well plates (Greiner, Longwood, FL) were precoated with a 0.001% (wt/vol) poly-L-lysine solution (Sigma-Aldrich, St. Louis, MO) for 1h at 37° C. Poly-L-lysine was removed and wells rinsed with RPMI before cell addition (75,000–150000 lymphocytes/well).

PBMCs were thawed in complete RPMI 1640 growth media supplemented with 10% FBS (v/v) and seeded at a concentration of 100000 cells/ well in triplicate wells per sample. Cells were left to adhere to the plate for 1 h at 37C in incubator with 95% humidity, 5% CO_2. Plates were then removed from incubator and then fixed at room temperature (RT) by slowly filling up the wells with 200 ul methanol/acetic acid (3/1, vol/vol) and incubated for 10 to 15 min. The solution was removed, and this was repeated 2 more times, leaving the last fixative volume up for a total of 1 h fixation. Plates were then moved to -20 until processed for high-throughput quantitative FISH (HT Q-FISH).

We performed HT Q-FISH as described before [46]. Briefly, the plates were removed from -20, the fixative solution removed, and the plates were dried on a hot plate at 37° C overnight, followed by rehydration with 200 µL of PBS. Cells were fixed with 200 µL of 4% formaldehyde in PBS for 2 min at room temperature (RT) and washed 3 times for 5 min with PBS. Prewarmed pepsin solution (100 mL of H_2O, 100 µL of 37% HCl [10.1 M HCl], and 100 mg of pepsin [Sigma-Aldrich; catalog no. P7000-25G]) was used to degrade cell walls for 15 min at 37° C followed by 2 washing steps of 5 min with 200 µL of PBS. The cells were then dehydrated with sequential 5-min 70%, 90%, and 100% ethanol steps. The plates were dried 1 h at 37° C. Next, 50 µL of the hybridization solution containing the Tel-Cy3 PNA probe was added to the plates (95 µL of 1 M Tris, pH 7.0, 812 µL of $MgCl_2$ solution [25 mM $MgCl_2$, 9 mM citric acid, 82 mM Na_2HPO_4], 6.65 mL of deionized formamide, 475 µL of blocking reagent [10 g of blocking reagent (Boehringer; catalog no. 1093 657) dissolved with heat in 100 mL of maleic acid buffer, pH 7.5 (100 mM maleic acid, 150 mM NaCl)], 1.28 mL of H_2O, and 190 µL of Tel-Cy3 PNA probe [5 µg lyophilized Cy3-(C3TA2)3 PNA probe (Panagene) diluted in 200 µL of H_2O]). Plates were then sealed with aluminum foil lids. The DNA was denatured by heating the plate on a hot plate at 85° C for 5 min and left to incubate for 2 h at RT in the dark. The plates were rinsed and washed in plate shaker with wash solution 1 (10 mM Tris-HCl pH 7, 70% Formamide, 0.10% BSA in H_2O) for 30 min, followed by 3 washes of 5 min each with wash solution 2 (TBS [Tris-buffered saline, pH 7.0] with 0.08% Tween 20). Nuclei staining was performed incubating for 10 min with TBST containing 1 µg/mL DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Life Technologies; catalog no. D-1306). Then the plates were washed 1 × 5 min with PBS and stored at 4° C in the dark. Images from the plate were then acquired by HT microscopy within 48 h.

High-throughput microscopy

Quantitative image acquisition was performed on an Opera High Content Screening System (PerkinElmer) 40×/0.9 N.A. water-immersion objective. UV and 561 nm excitation wavelengths were used to detect DAPI and Cy3 telomeric signals, respectively and 60

independent images were captured at different positions of each well. Images were analyzed with Acapella Image analysis software (PerkinElmer). Data were analyzed with SPSS (IBM) and Excel (Microsoft). Telomere fluorescence values were converted into kilobases by external calibration with the CCRF-CEM (7.5 kb), L5178Y-S (10.2 kb), L5178Y-R (79.7 kb) and Jurkat (11.5 kb) cell lines.

Criteria for the diagnosis of COVID-19

Depending on the clinical features of COVID-19, patients are generally divided as mild, moderate, severe and acute.

1. Mild COVID-19: low-grade fever, cough, malaise, rhinorrhea, sore throat with or without hemoptysis, nausea, vomiting, diarrhea, but without any radiological features of pneumonia and absence of mental changes.

2. Moderate COVID-19: fever, respiratory symptoms including dry cough and shortness of breath that may emerge along with the radiological features.

3. Severe COVID-19: dyspnea, respiratory frequency ≥30/minute, blood oxygen saturation ≤93%, PaO2/FiO2 ratio <300, and/or lung infiltrates >50% of the lung field within 24–48 h.

4. Acute COVID-19: usually develops after 7 days in patients with mild/moderate/severe COVID-19 with features of Acute respiratory distress syndrome (ARDS) requiring mechanical ventilation along with presence of multiorgan dysfunction failure, metabolic acidosis and coagulation dysfunction.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

MAB had the original idea and secured funding. MAB and PM supervised research and wrote the paper. RSV, AGC and PM analyzed the data and performed experiments. AZG provided blood samples and clinical information.

CONFLICTS OF INTEREST

MAB is founder and holds shares of Life Length SL, a Biotech company that measures telomere length for biomedical uses.

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REFERENCES

1. Han C, Duan C, Zhang S, Spiegel B, Shi H, Wang W, Zhang L, Lin R, Liu J, Ding Z, Hou X. Digestive symptoms in COVID-19 patients with mild disease severity: clinical presentation, stool viral RNA testing, and outcomes. Am J Gastroenterol. 2020; 115:916–23. https://doi.org/10.14309/ajg.0000000000000664 PMID:32301761

2. Li YC, Bai WZ, Hashikawa T. The neuroinvasive potential of SARS-CoV2 may play a role in the respiratory failure of COVID-19 patients. J Med Virol. 2020; 92:552–55. https://doi.org/10.1002/jmv.25728 PMID:32104915

3. Tam CF, Cheung KS, Lam S, Wong A, Yung A, Sze M, Lam YM, Chan C, Tsang TC, Tsui M, Tse HF, Siu CW. Impact of Coronavirus Disease 2019 (COVID-19) Outbreak on ST-Segment-Elevation Myocardial Infarction Care in Hong Kong, China. Circ Cardiovasc Qual Outcomes. 2020. https://doi.org/10.1161/CIRCOUTCOMES.120.006631 PMID:32182131

4. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 2020; 181:271–80.e8. https://doi.org/10.1016/j.cell.2020.02.052 PMID:32142651

5. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. Cell. 2020; 181:281–92.e6. https://doi.org/10.1016/j.cell.2020.02.058 PMID:32155444

6. Wan Y, Shang J, Graham R, Baric RS, Li F. Receptor recognition by the novel coronavirus from Wuhan: an analysis based on decade-long structural studies of SARS coronavirus. J Virol. 2020; 94:e00127–20.

https://doi.org/10.1128/JVI.00127-20
PMID:31996437

7. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020; 367:1260–63. https://doi.org/10.1126/science.abb2507 PMID:32075877

8. Zhao Y, Zhao Z, Wang Y, Zhou Y, Ma Y, Zuo W. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov. bioRxiv, 2020.

9. Crackower MA, Sarao R, Oudit GY, Yagil C, Kozieradzki I, Scanga SE, Oliveira-dos-Santos AJ, da Costa J, Zhang L, Pei Y, Scholey J, Ferrario CM, Manoukian AS, et al. Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature. 2002; 417:822–28. https://doi.org/10.1038/nature00786 PMID:12075344

10. Danilczyk U, Penninger JM. Angiotensin-converting enzyme II in the heart and the kidney. Circ Res. 2006; 98:463–71. https://doi.org/10.1161/01.RES.0000205761.22353.5f PMID:16514079

11. Ding Y, He L, Zhang Q, Huang Z, Che X, Hou J, Wang H, Shen H, Qiu L, Li Z, Geng J, Cai J, Han H, et al. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. J Pathol. 2004; 203:622–30. https://doi.org/10.1002/path.1560 PMID:15141376

12. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, Zou W, Zhan J, Wang S, Xie Z, Zhuang H, Wu B, Zhong H, et al. Multiple organ infection and the pathogenesis of SARS. J Exp Med. 2005; 202:415–24. https://doi.org/10.1084/jem.20050828 PMID:16043521

13. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol. 2004; 203:631–37. https://doi.org/10.1002/path.1570 PMID:15141377

14. Zhang H, Penninger JM, Li Y, Zhong N, Slutsky AS. Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: molecular mechanisms and potential therapeutic target. Intensive Care Med. 2020; 46:586–90. https://doi.org/10.1007/s00134-020-05985-9 PMID:32125455

15. Ling Y, Xu SB, Lin YX, Tian D, Zhu ZQ, Dai FH, Wu F, Song ZG, Huang W, Chen J, Hu BJ, Wang S, Mao EQ, et al. Persistence and clearance of viral RNA in 2019 novel coronavirus disease rehabilitation patients. Chin Med J (Engl). 2020; 133:1039–43. https://doi.org/10.1097/CM9.0000000000000774 PMID:32118639

16. Young BE, Ong SW, Kalimuddin S, Low JG, Tan SY, Loh J, Ng OT, Marimuthu K, Ang LW, Mak TM, Lau SK, Anderson DE, Chan KS, et al, and Singapore 2019 Novel Coronavirus Outbreak Research Team. Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. JAMA. 2020; 323:1488–94. https://doi.org/10.1001/jama.2020.3204 PMID:32125362

17. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

18. Tian S, Hu W, Niu L, Liu H, Xu H, Xiao SY. Pulmonary pathology of early-phase 2019 novel coronavirus (COVID-19) pneumonia in two patients with lung cancer. J Thorac Oncol. 2020; 15:700–04. https://doi.org/10.1016/j.jtho.2020.02.010 PMID:32114094

19. Ruf BR, Knuf M. The burden of seasonal and pandemic influenza in infants and children. Eur J Pediatr. 2014; 173:265–76. https://doi.org/10.1007/s00431-013-2023-6 PMID:23661234

20. Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos AM, Posey DL, Klimov A, Lindstrom SE, Balish A, Medina MJ, Wallis TR, et al, and Influenza Special Investigations Team. Influenza-associated deaths among children in the United States, 2003-2004. N Engl J Med. 2005; 353:2559–67. https://doi.org/10.1056/NEJMoa051721 PMID:16354892

21. Finelli L, Fiore A, Dhara R, Brammer L, Shay DK, Kamimoto L, Fry A, Hageman J, Gorwitz R, Bresee J, Uyeki T. Influenza-associated pediatric mortality in the United States: increase of Staphylococcus aureus coinfection. Pediatrics. 2008; 122:805–11. https://doi.org/10.1542/peds.2008-1336 PMID:18829805

22. Fleming DM, Pannell RS, Cross KW. Mortality in children from influenza and respiratory syncytial virus. J Epidemiol Community Health. 2005; 59:586–90. https://doi.org/10.1136/jech.2004.026450 PMID:15965143

23. Nair H, Brooks WA, Katz M, Roca A, Berkley JA, Madhi SA, Simmerman JM, Gordon A, Sato M, Howie S,

Krishnan A, Ope M, Lindblade KA, et al. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. Lancet. 2011; 378:1917–30. https://doi.org/10.1016/S0140-6736(11)61051-9 PMID:22078723

24. Sachedina N, Donaldson LJ. Paediatric mortality related to pandemic influenza a H1N1 infection in England: an observational population-based study. Lancet. 2010; 376:1846–52. https://doi.org/10.1016/S0140-6736(10)61195-6 PMID:21030071

25. Bellon M, Nicot C. Telomere dynamics in immune senescence and exhaustion triggered by chronic viral infection. Viruses. 2017; 9:289. https://doi.org/10.3390/v9100289 PMID:28981470

26. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet. 2005; 6:611–22. https://doi.org/10.1038/nrg1656 PMID:16136653

27. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013; 153:1194–217. https://doi.org/10.1016/j.cell.2013.05.039 PMID:23746838

28. Blackburn EH. Structure and function of telomeres. Nature. 1991; 350:569–73. https://doi.org/10.1038/350569a0 PMID:1708110

29. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev. 2005; 19:2100–10. https://doi.org/10.1101/gad.1346005 PMID:16166375

30. Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat Rev Cancer. 2011; 11:161–76. https://doi.org/10.1038/nrc3025 PMID:21346783

31. Olovnikov AM. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J Theor Biol. 1973; 41:181–90. https://doi.org/10.1016/0022-5193(73)90198-7 PMID:4754905

32. Watson JD. Origin of concatemeric T7 DNA. Nat New Biol. 1972; 239:197–201. https://doi.org/10.1038/newbio239197a0 PMID:4507727

33. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990; 345:458–60. https://doi.org/10.1038/345458a0 PMID:2342578

34. Vera E, Bernardes de Jesus B, Foronda M, Flores JM, Blasco MA. The rate of increase of short telomeres predicts longevity in mammals. Cell Rep. 2012; 2:732–7. https://doi.org/10.1016/j.celrep.2012.08.023 PMID:23022483

35. Whittemore K, Vera E, Martínez-Nevado E, Sanpera C, Blasco MA. Telomere shortening rate predicts species life span. Proc Natl Acad Sci USA. 2019; 116:15122–27. https://doi.org/10.1073/pnas.1902452116 PMID:31285335

36. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. A DNA damage checkpoint response in telomere-initiated senescence. Nature. 2003; 426:194–98. https://doi.org/10.1038/nature02118 PMID:14608368

37. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in tetrahymena extracts. Cell. 1985; 43:405–13. https://doi.org/10.1016/0092-8674(85)90170-9 PMID:3907856

38. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell. 1997; 91:25–34. https://doi.org/10.1016/s0092-8674(01)80006-4 PMID:9335332

39. Flores I, Cayuela ML, Blasco MA. Effects of telomerase and telomere length on epidermal stem cell behavior. Science. 2005; 309:1253–56. https://doi.org/10.1126/science.1115025 PMID:16037417

40. Herrera E, Samper E, Martín-Caballero J, Flores JM, Lee HW, Blasco MA. Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J. 1999; 18:2950–60. https://doi.org/10.1093/emboj/18.11.2950 PMID:10357808

41. Lee HW, Blasco MA, Gottlieb GJ, Horner JW 2nd, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. Nature. 1998; 392:569–74. https://doi.org/10.1038/33345 PMID:9560153

42. Armanios M, Blackburn EH. The telomere syndromes. Nat Rev Genet. 2012; 13:693–704. https://doi.org/10.1038/nrg3246 PMID:22965356

43. Povedano JM, Martinez P, Flores JM, Mulero F, Blasco MA. Mice with pulmonary fibrosis driven by telomere dysfunction. Cell Rep. 2015; 12:286–99. https://doi.org/10.1016/j.celrep.2015.06.028 PMID:26146081

44. Povedano JM, Martinez P, Serrano R, Tejera Á, Gómez-López G, Bobadilla M, Flores JM, Bosch F, Blasco MA.

Therapeutic effects of telomerase in mice with pulmonary fibrosis induced by damage to the lungs and short telomeres. Elife. 2018; 7:e31299. https://doi.org/10.7554/eLife.31299 PMID:29378675

45. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 2009; 37:e21. https://doi.org/10.1093/nar/gkn1027 PMID:19129229

46. Canela A, Vera E, Klatt P, Blasco MA. High-throughput telomere length quantification by FISH and its application to human population studies. Proc Natl Acad Sci USA. 2007; 104:5300–05. https://doi.org/10.1073/pnas.0609367104 PMID:17369361

47. Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. Accelerated telomere shortening in response to life stress. Proc Natl Acad Sci USA. 2004; 101:17312–15. https://doi.org/10.1073/pnas.0407162101 PMID:15574496

48. Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. Obesity, cigarette smoking, and telomere length in women. Lancet. 2005; 366:662–64. https://doi.org/10.1016/S0140-6736(05)66630-5 PMID:16112303

49. Zhang YZ, Holmes EC. A Genomic Perspective on the Origin and Emergence of SARS-CoV-2. Cell. 2020; 181:223–27. https://doi.org/10.1016/j.cell.2020.03.035 PMID:32220310

50. Bernardes de Jesus B, Vera E, Schneeberger K, Tejera AM, Ayuso E, Bosch F, Blasco MA. Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer. EMBO Mol Med. 2012; 4:691–704. https://doi.org/10.1002/emmm.201200245 PMID:22585399

51. Martínez P, Blasco MA. Telomere-driven diseases and telomere-targeting therapies. J Cell Biol. 2017; 216:875–87. https://doi.org/10.1083/jcb.201610111 PMID:28254828

Research Paper

Fighting the storm: could novel anti-TNFα and anti-IL-6 *C. sativa* cultivars tame cytokine storm in COVID-19?

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ABSTRACT

The main aspects of severe COVID-19 disease pathogenesis include hyper-induction of proinflammatory cytokines, also known as 'cytokine storm', that precedes acute respiratory distress syndrome (ARDS) and often leads to death. COVID-19 patients often suffer from lung fibrosis, a serious and untreatable condition. There remains no effective treatment for these complications. Out of all cytokines, TNFα and IL-6 play crucial roles in cytokine storm pathogenesis and are likely responsible for the escalation in disease severity. These cytokines also partake in the molecular pathogenesis of fibrosis. Therefore, new approaches are urgently needed, that can efficiently and swiftly downregulate TNFα, IL-6, and the inflammatory cytokine cascade, in order to curb inflammation and prevent fibrosis, and lead to disease remission.

Cannabis sativa **has been proposed to modulate gene expression and inflammation and is under investigation for several potential therapeutic applications against autoinflammatory diseases and cancer. Here, we hypothesized that the extracts of novel *C. sativa* cultivars may be used to downregulate the expression of pro-inflammatory cytokines and pathways involved in inflammation and fibrosis.**

Initially, to analyze the anti-inflammatory effects of novel *C. sativa* cultivars, we used a well-established full thickness human 3D skin artificial EpiDermFTTM tissue model, whereby tissues were exposed to UV to induce inflammation and then treated with extracts of seven new cannabis cultivars. We noted that out of seven studied extracts of novel *C. sativa* cultivars, three (#4, #8 and #14) were the most effective, causing profound and concerted down-regulation of COX2, TNFα, IL-6, CCL2, and other cytokines and pathways related to inflammation and fibrosis. These data were further confirmed in the WI-38 lung fibroblast cell line model. Most importantly, one of the tested extracts had no effect at all, and one exerted effect that may be deleterious, signifying that careful cannabis cultivar selection must be based on thorough pre-clinical studies. The observed pronounced inhibition of TNFα and IL-6 is the most important finding, because these molecules are currently considered to be the main targets in COVID-19 cytokine storm and ARDS pathogenesis.

Novel anti-TNFα and anti-IL-6 cannabis extracts can be useful additions to the current anti-inflammatory regimens to treat COVID-19, as well as various rheumatological diseases and conditions, and 'inflammaging' - the inflammatory underpinning of aging and frailty.

INTRODUCTION

To date, raging pandemic of COVID-19 disease caused by the SARS-CoV2 virus has affected over 80 million people and claimed over 1,750,000 lives worldwide. SARS-CoV2 has human-human transmission and spreads easily via airborne and contact routes; its R_0 is currently estimated to be 2-2.5 [1]. COVID-19 has a rather broad spectrum of clinical manifestations, ranging from asymptomatic, to mild flu-like disease, to pneumonia, that in some cases can further progress to acute respiratory distress syndrome (ARDS), major organ failure and death. Approximately 20% of COVID-19 cases are serious or severe, and death rate is currently estimated to be around 10%. While elderly and individuals with pre-existing conditions are among the most affected, it has recently become apparent that COVID-19 affects all age groups.

Along with virus levels, the key aspects of the severe COVID-19 disease pathogenesis include increasing hyper-induction of proinflammatory cytokines, which is also known as 'cytokine storm' that precedes acute respiratory distress syndrome (ARDS) [2, 3]. It is now well-established that the severity of COVID-19 is due to the host immune response [4] and that the cytokine storm, a host-mediated response, is a key feature of immunopathogenesis of COVID-19 infection [4, 5].

Overall, various plasma cytokines and chemokines were reported to be deregulated in COVID-19 patients; these include TNF-α, interleukins (IL-1, IL-2, IL- 4, IL-7, IL-10, IL-12, IL-13, IL-17), macrophage colony-stimulating factor (MCSF), IP-10, MCP-1 (C-C motif chemokine 2, CCL2), MIP-1α, hepatocyte growth factor (HGF), IFN-γ, CCL3, CCL5 and many others [6]. Cytokine levels correlate with disease severity [7]. Patients with moderate COVID-19 disease had elevated levels of TNFα and IL-6, and in severe COVID-19 cases the production of IL-6 and TNF-α and other cytokines was profoundly increased [7]. Moreover, patients requiring ICU admission had higher levels of IL-6, IL-2, IL-7, IL-10, GCSF, IP10, CCL2, MIP1A, and TNFα than did those not requiring ICU admission, suggesting that the cytokine storm was important in COVID-19 pathogenesis [8, 9].

Of the cytokine milieu, TNFα and IL-6 play key roles in cytokine storm and are likely to be responsible for the escalation in disease severity [10–12]. TNFα is an inflammatory cytokine that stimulates and maintains cellular activation and migration of leukocytes to inflammatory sites. TNF acts by binding to its receptors (TNFR) that are located throughout the body. Interaction of TNF with receptors causes increased expression of other cytokines (IL-1 and IL-6) and chemokines, which, in turn, activate leukocytes, suppresses regulatory T cells, causes production of MMP proteins which degrade tissues and induce apoptosis [13]. IL-6 is another important player in the acute host response to infection whereby it promotes inflammation, immune reactions, and hematopoiesis. Long-term elevation of IL-6 levels maintains chronic inflammation and autoimmunity, making IL-6 one of the main druggable targets in autoinflammatory and autoimmune disorders [14].

Even though TNFα- and IL-6-mediated cytokine storm and ARDS have been previously well-documented in SARS, MERS, as well as in severe cases of influenza [3, 15], there still is no effective treatment for this grievous complication. Therefore, new approaches are urgently needed that can efficiently and swiftly block TNFα, IL-6 and inflammatory cytokine cascades and thus curb inflammation and lead to disease remission.

Furthermore, COVID-19 convalescents face a long recovery and may be at risk of developing pulmonary fibrosis (PF), a debilitating complication that is very hard to treat [16]. Mechanisms of PF are not fully understood, albeit it has been established that inflammatory cytokines and chemokines, such as IL-1, IL-6, TNFα, C-C motif chemokines are important in its etiology [5, 17]. New therapies are much needed to prevent and mitigate pulmonary fibrosis complications in COVID-19 patients. Since COVID-19, and especially ARDS patients are extremely weak and vulnerable, it would be crucial that novel anti-cytokine storm and anti-fibrosis therapies have minimal side effects.

Cannabis sativa has been proposed to modulate gene expression and inflammation and is under investigation for several potential therapeutic applications against autoinflammatory diseases and cancer. Therefore, we hypothesized that extracts of novel *C. sativa* cultivars may be used to downregulate expression of pro-inflammatory cytokines and pathways involved in inflammation and fibrosis.

RESULTS

Cannabis extracts affect the expression of inflammation-related genes and proteins in the EpiDermFT model

For the initial analysis of the anti-inflammatory effects of novel *C. sativa* cultivars, we used a well-established full thickness human 3D skin artificial EpiDermFT™ tissue model, whereby tissues were exposed to UV to induce inflammation and then treated with extracts of seven new cannabis cultivars. Upon original screening of over 200 extracts, seven extracts of cultivars #4, #6,

#8, #12, #13, #14, #15, were identified for further analysis.

Analysis of global gene expression profiling revealed that 5 new extracts strongly down-regulated expression of interleukins, pro-inflammatory cytokines, C-C motif chemokines and C–X–C subfamily cytokines involved in ADRS and other autoinflammatory conditions (padj<0.05) (Figure 1 and Table 1).

TNFα and IL-6: Application of the extracts # 4, #6, #8 and #14 down-regulated both TNFα and IL-6. Extract #13 downregulated TNFα but not IL-6. Interestingly, extract #12 upregulated the expression of IL-6 and IL-23A, pro-inflammatory chemokines, and down-regulated the expression of anti-inflammatory IL-37. Application of extract#15 did not result in any statistically significant gene expression changes (Figure 1 and Table 1).

COX2: Moreover, extracts #4, #6, #8, #13 and #14 significantly down-regulated the expression of prostaglandin-endoperoxide synthase 2 (PTGS2) gene that encodes for cyclooxygenase 2 (COX2). Extract #15 had no effects on PTGS2 levels, whereas extract #12 caused an upregulation of PTGS2 expression (Figure 2).

We further explored the effects of *C. sativa* extracts on the levels of IL-6 and COX2 proteins using western immunoblotting, and found that all extracts, except #15, downregulated UV-induced IL-6 expression and all extracts downregulated UV-triggered COX2 induction (Figure 3). Interestingly, application of extract #12 downregulated IL-6 on the protein level, but not on the level of the transcript. This is an interesting finding that may suggest the presence of post-transcriptional regulation of IL-6 expression via small interfering RNAs and the potential effects of cannabis extracts on these processes.

IL-1, IL-17, IL-23: Along with two key regulators of cytokine storm – TNFα and IL-6, *C. sativa* extracts also affected the levels of other key pro-inflammatory interleukins – IL-1, IL-17, IL-23 (Figure 1 and Table 1). Here, we found that extracts #4 and #8 downregulated both IL-1α and IL-1β (Figure 1 and Table 1). Further, extracts #4, #6, #8, #13 and #14 downregulated, while extract #12 upregulated IL-23A, a member of the IL-12

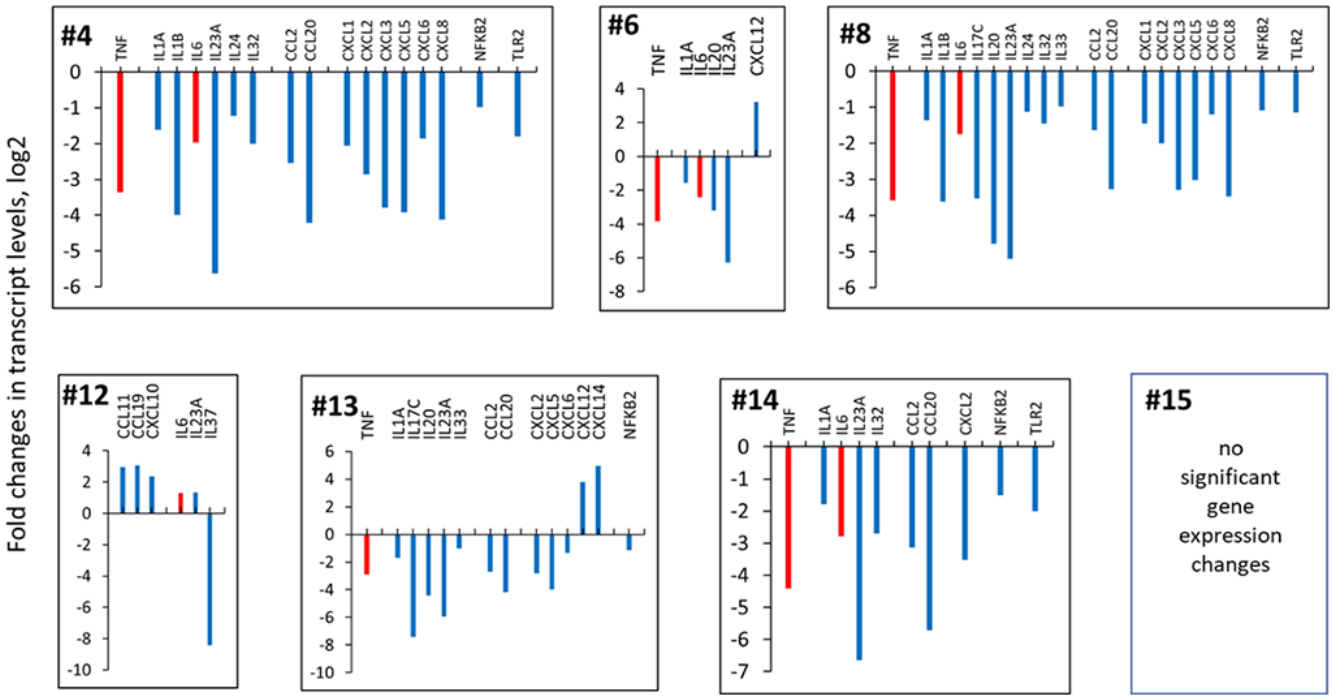

Figure 1. Effects of novel *C. sativa* extracts on the levels of pro-inflammatory cytokines. To induce inflammation, human 3D EpiDermFT tissues were exposed to UV. Upon exposure, tissues were incubated with extracts or vehicle (DMSO) for 24 h. Three tissues were used for each condition. The differences between all experimental groups were examined using the likelihood ratio test (LRT) test implemented in DESeq2. The reduced model included the intercept and the full model was the experimental group (Cannabis extracts and controls). Multiple comparisons adjustment of p-values was done using Benjamini-Hochberg procedure [63]. Specific comparisons between groups were done using *results()* function with *contrast* argument specified. Genes with adjusted p-values below 0.05 were considered significant. Data are shown as log 2 fold changes as compared to UV-induced tissues. All changes shown here are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

Table 1. Effects of cannabis cultivars on the levels inflammation and fibrosis-related genes human 3D EpiDermFT tissues, as studied by the global transcriptome profiling using RNAseq.

LINE	#4	#8	#14	#13	#6	#12	#15
Inflammation- and fibrosis-related genes							
TNF	**-3.4**	**-3.6**	**-4.4**	**-2.9**	**-3.9**		
IL1A	-1.6	-1.4	-1.8	-1.7	-1.6		
IL1B	-4.0	-3.6			-2.4		
IL6	**-2.0**	**-1.8**	**-2.8**		**-2.4**	**1.3**	
IL17C		-3.5		-7.4			
IL20		-4.8		-4.4	-3.2		
IL23A	-5.6	-5.2	-6.7	-6.0	-6.3	1.3	
IL24	-1.2	-1.1					
IL32	-2.0	-1.5	-2.7		3.2		
IL33		-1.0		-1.0			
IL37						-8.4	
CCL2	-2.5	-1.6	-3.1	-2.7			
CCL20	-4.2	-3.3	-5.7	-4.2			
CXCL1	-2.1	-1.4					
CXCL2	-2.9	-2.0	-3.5	-2.8			
CXCL3	-3.8	-3.3					
CXCL5	-3.9	-3.0		-4.0			
CXCL6	-1.8	-1.2		-1.3			
CXCL8	-4.1	-3.5					
CXCL10						2.3	
CXCL12				3.8	3.2		
CXCL14				5.0			
NFKB2	-1.0	-1.1	-1.5	-1.1			
PTGS2	-3.3	-2.5	-3.7	-2.9	-3.3	1.6	
TLR2	-1.8	-1.2	-2.0				
Fibrosis-related genes							
MMP1	-2.7	-1.8					
MMP3		-1.8					
MMP7		2.7		3.6	2.8		
MMP8		-1.5		-2.0			
MMP10	-1.7	-1.7	-1.5				
MMP11				3.2	2.9		
MMP19		-1.0		-1.1			
WNT2	-2.2	-1.5	-2.1	-1.5	-2.2		
WNT5A	-1.5	-1.2	-1.5	-1.4	-1.3		
FZD4	-1.2						
ICAM1	-1.5	-1.4	-2.2		-1.8		
ICAM5	-1.6	-2.0					

Data are shown as log 2 fold changes as compared to UV-induced tissues. All changes shown here are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

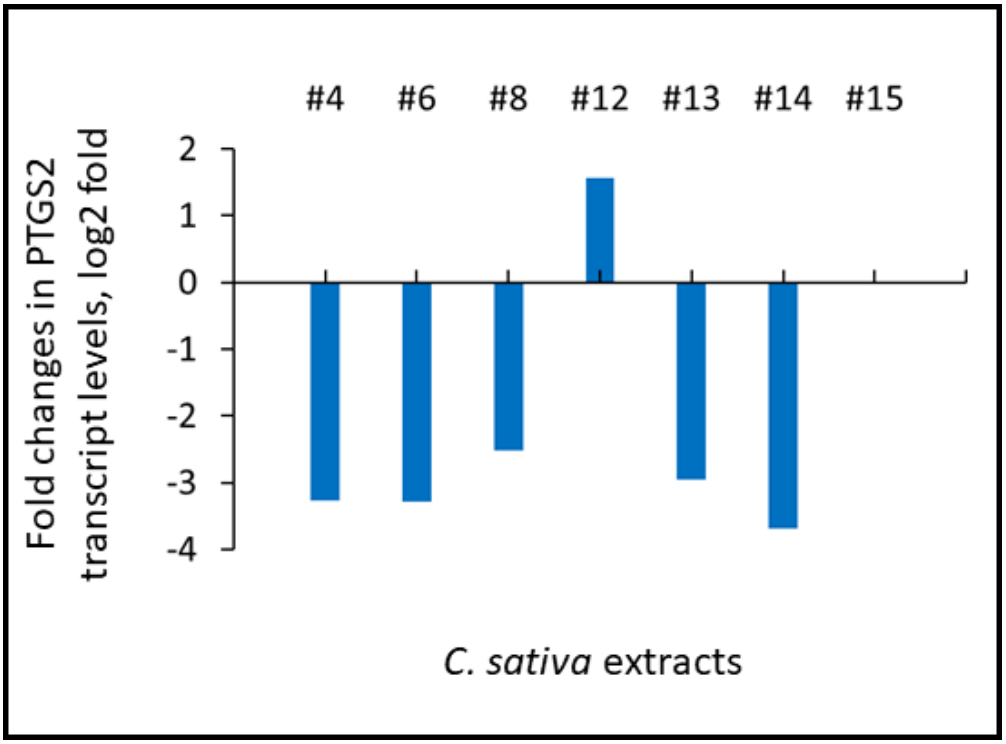

Figure 2. Effects of novel *C. sativa* extracts on the levels of PTGS2 gene expression as studied by the global transcriptome profiling using RNAseq. Induction of inflammation and treatments were described in the legend to Figure 1. Data are shown as log 2 fold changes as compared to UV-induced tissues. All changes shown here are statistically significant, p adj <0.05, ANOVA-like analysis and pair-wise comparison.

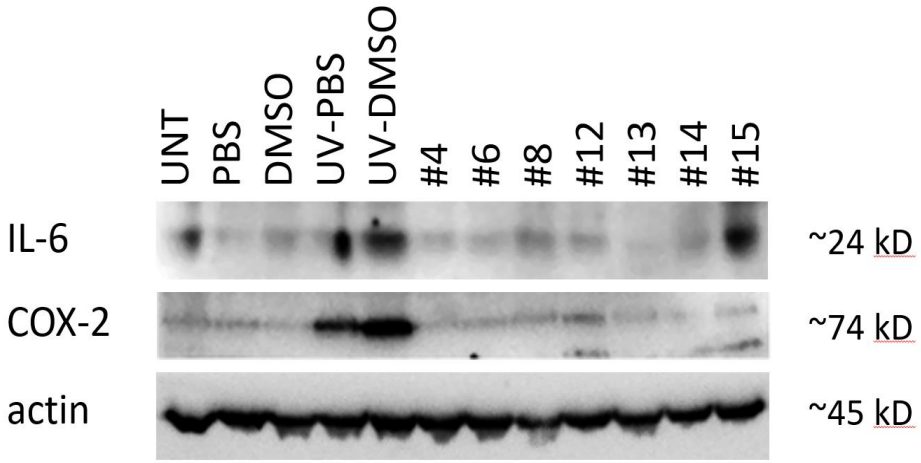

Figure 3. Effects of novel *C. sativa* extracts on the levels of IL-6 and COX2 in human EpiDerm FT tissues. To induce inflammation, tissues were exposed to UV. Upon exposure, tissues were incubated with extracts or vehicle (DMSO) for 24 h. Three tissues were used for each condition. Western blot analysis was performed using antibodies against IL-6 and COX2 as detailed in the Methods section. "UNT" – untreated tissues; "PBS" - 15 µl of 30% glycerol in PBS was applied to the tissues and no exposure was done; "DMSO" - 15 µl of DMSO (0.017% in 30% glycerol-PBS) was applied to the tissues and no exposure was done; "UV-PBS" - tissues were exposed to UV and 15 µl of 30% glycerol-PBS was applied to them; "UV-DMSO" – tissues were exposed to UV and 15 µl of DMSO (0.017% in 30% glycerol-PBS) was applied to them; "#4" through "#15" - tissues were exposed to UV and 15 µl of extracts in DMSO was applied to them.

family of cytokines with pro-inflammatory properties [18]. Extracts #8 and #13 downregulated IL-17C, a pro-inflammatory cytokine and a member of IL-17 family, that, together with IL-23 mediates inflammation in psoriasis, psoriatic arthritis, and ankylosing spondylitis [19].

TLR: Three extracts, #4, #8 and #14 downregulated the levels of Toll-like receptor 2 (TLR2), which has been implicated in numerous inflammatory diseases [20], including pulmonary diseases and ARDS [21].

NFKB2: In addition, extracts #4, #8, #13 and #14 significantly down-regulated the expression of NFKB2 gene, which has been often referred to as a prototypical proinflammatory signaling pathway. NF-κB is usually upregulated by IL-1 and TNFα, and play important roles in the expression of other proinflammatory genes [22].

Cannabis extracts affect the levels of fibrosis-related genes in the EpiDermFT model

We next looked at the effect of cannabis extracts on the levels of fibrosis-related mRNAs. Global gene expression profiling analysis revealed that extracts #4, #8, #13 and #14 downregulated CCL2, also known as MCP-1 (Figure 1 and Table 1), which is an important hallmark of fibrosis, and has been indicated as a potential druggable anti-fibrotic target [23]. Several extracts down-regulated MMPs (Table 1).

Extracts #4, #6, #8, #14 and #13 also down-regulated WNT2 and WNT5a. WNT signaling alterations have been linked to pathogenesis of a variety of diseases and conditions, including pulmonary fibrosis [24, 25]. Furthermore, extracts also affected the levels of iCAM1 and iCAM5 genes.

One more important pro-fibrotic protein is CXCL12, and its down-regulation was shown to dampen fibrocyte recruitment and collagen deposition [26]. In our study, extracts #6 and #13, along with down-regulation of numerous pro-inflammatory cytokines, upregulated CXCL12.

In-depth analysis reveals pathways affected by cannabis extracts in EpiDermFT tissues

Having seen cannabis extract-induced changes in pro-inflammatory and pro-fibrotic genes, we further conducted an in-depth analysis of the effects of the extracts on global signalome using Pathview Bioconductor platform. We found that extracts # 4, #8, #14 significantly down-regulated cytokine-cytokine receptor interaction pathway, rheumatoid arthritis pathway, chemokine signalling, Toll-like receptor signalling, JAK-STAT signalling and other pathways involved in inflammation, immunity and autoimmunity, as well as tissue remodeling and fibrosis. Contrarily, extract #12 upregulated these pathways (Table 2 and Supplementary Figure 1).

Correlation between extract composition and molecular effects

Overall, our study revealed that cannabis extracts exerted different effects on the 3D tissue inflammation model - some profoundly down-regulated pro-inflammatory cytokines and pro-fibrotic molecules, some affected only several key cytokines, some did not cause any significant changes at all (extract #15), while extract #12 promoted expression of pro-inflammatory genes. This is a very important finding that shows that cannabis is non-generic. Indeed, cultivars have unique profiles of cannabinoids and terpenes that can potentiate each other [27], and hence extracts of different cultivars may have different medicinal properties, even though the ratios of major cannabinoids (THC and CBD) may be similar. Hence each *C. sativa* cultivar has to be thoroughly evaluated for its medicinal properties.

To find whether there was any correlation between the level of cannabinoids in the extracts and the efficiency of the extracts in downregulation of inflammation- and fibrosis-related genes, we analyzed the concentration of THC, CBD, CBGA and CBN in flowers and in the extracts (Table 3). We then ranked the efficiency of the extracts by summing up the values for downregulation of all genes in Table 1. Extracts ranked #4, #8, #14, #13, #6, #12, #15, with #4 being the most efficient and #15 the least efficient. We then correlated the concentration of individual cannabinoids with the efficiency of extracts. We found weak positive (0.24) correlation with the level of total THC (THC and THCA) and weak negative correlation with total CBD (CBD and CBD-A), CBGA and CBN, -0.29, -0.32 and -0.32, respectively. We next analyzed the presence and the concentration of terpenes in three extracts, #8, #6 and #12, with #8 being the best (and equal to #4), #6 being an average, and #12 one of the worst. We found that extract #8 was dominant in β-caryophyllene and caryophyllene oxide, while extract #6 was dominant in α-bisabolol and guaiol, and extract #12 in linalool and guaiol. Further studies are needed to establish the roles of terpenes and their effects on inflammation.

Cannabis extracts inhibit COX-2 and IL-6 levels in WI-38 lung fibroblasts

While the observed effects were clearly interesting and intriguing, they called for more studies to analyze these effects in a lung model system. Thus, having seen

Table 2. Pathways most significantly deregulated in EFT-400 tissues upon treatments with extracts of new *C. sativa* cultivars #4. #14, #8 and #12.

KEGGID	Pvalue	Term	Genes
Cultivar #4			
DOWNREGULATED PATHWAYS			
5323	1.79E-11	Rheumatoid arthritis	6374;1437;6364;51561;3553;2919;6347;4312;6372;3569;3576;2321;3383;7097;3552;3689;7124
4060	4.22E-11	Cytokine-cytokine receptor interaction	6374;1437;6364;51561;2921;3553;2919;3976;57007;2920;6347;6372;3569;3575;7850;3576;2321;3552;7133;3082;7124;84957;1440;11009;23529
4514	2.51E-06	Cell adhesion molecules (CAMs)	25945;4897;80380;3383;3696;214;3689;1364;257194;1366;29126;23562;3134
4062	3.82E-05	Chemokine signaling pathway	6374;6364;2921;2919;2920;6347;6372;3055;2791;3576;4792;5908;57580;5604;114
4630	0.000634346	Jak-STAT signaling pathway	3598;1437;51561;3976;3569;3575;6775;81848;1440;11009;23529
4210	0.000993993	Apoptosis	3553;330;3656;3552;4792;11213;5533;637;7124
4620	0.008055248	Toll-like receptor signaling pathway	3553;3569;3576;7097;4792;5604;7124
4660	0.019535451	T cell receptor signaling pathway	1437;4792;5533;4773;5604;7124;4794
Cultivar #14			
DOWNREGULATED PATHWAYS			
4621	5.24E-06	NOD-like receptor signaling pathway	6347;2920;3569;7128;330;8767;7124;4792
4060	9.06E-06	Cytokine-cytokine receptor interaction	51561;6347;3976;2920;57007;3569;6364;7124;7133;23529;7850;84957;3552
5323	4.01E-05	Rheumatoid arthritis	51561;6347;3383;3569;6364;7124;3552;7097
4514	0.000993116	Cell adhesion molecules (CAMs)	3383;80380;25945;214;4897;1364;257194
4620	0.010832243	Toll-like receptor signaling pathway	3569;7124;4792;5606;7097
4062	0.04389252	Chemokine signaling pathway	6347;2920;3055;2791;6364;4792
4210	0.003683526	Apoptosis	330;7124;4792;3656;5533;3552
Cultivar #8			
DOWNREGULATED PATHWAYS			
4060	1.45E-13	Cytokine-cytokine receptor interaction	1440;6364;2921;3976;3553;51561;6374;2920;3589;57007;650;2919;6347;1437;3624;3576;3569;50604;3552;6372;3082;7124;51330;7133;84957;11009;23529
5323	7.81E-12	Rheumatoid arthritis	6364;3553;51561;6374;3589;4312;2919;6347;1437;3576;3569;3552;6372;3383;7097;7124;3689
4630	9.93E-05	Jak-STAT signaling pathway	1440;3976;51561;3589;3598;1437;3569;50604;81848;6775;11009;23529
4514	0.000889035	Cell adhesion molecules (CAMs)	25945;23562;4897;80380;3383;257194;3696;3689;3134
4062	0.000939189	Chemokine signaling pathway	6364;2921;6374;2920;2919;6347;2791;3576;6372;4792;9564;57580
4620	0.005637004	Toll-like receptor signaling pathway	3553;3576;3569;4792;7097;148022;7124
4210	0.010118422	Apoptosis	3553;3656;3552;4792;7124;330;5533
Cultivar #12			
UPREGULATED PATHWAYS			
4060	0.000239246	Cytokine-cytokine receptor interaction	3976;6376;6356;650;4982;7852;3627;6363;8995;3569;51561
4010	0.003164277	MAPK signaling pathway	1850;2353;1843;3164;2872;3725;3727;11221;1647;330

			3;22808
5323	0.008471825	Rheumatoid arthritis	2353;3725;5228;3569;51561
4512	0.045361608	ECM-receptor interaction	22801;1311;1301;1281
4510	0.047132189	Focal adhesion	80310;22801;3725;1311;1301;5228;1281
4620	0.047386067	Toll-like receptor signaling pathway	2353;3725;3627;3569

Pathways were generated using KEGG and rendered by Pathview. Details are provided in Materials and Methods.

Table 3. Level of single and total cannabinoids in flowers and extracts of selected *C. sativa* cultivars.

Flowers	**Total THC, %**	**Total CBD, %**	**CBGA, %**	**CBN, %**	**TOTAL Cannabinoids**
#4	14.7	0.76	0.1	0.06	15.62
#6	4.43	9.61	1.5		15.54
#8	14.72	0.14	0.22	0.02	15.1
#12	20.13	0.59	0.45	0.05	21.22
#13	16.49	0.16	0.17	0.03	16.85
#14	21.5	1.35	1.02		23.87
#15	14.57	0.46	0.1	0.14	15.13
Extracts	**Total THC, %**	**Total CBD, %**	**CBGA, %**	**CBN, %**	**TOTAL Cannabinoids**
#4	33.6	1.72	0.32	0.14	35.78
#6	10.3	23.4	3.4	0.1	37.1
#8	32.5	0.33	0.49	0.05	33.37
#12	43.2	1.8	0.92	0.12	46.04
#13	38.5	1.2	0.39	0.12	40.21
#14	44.3	1.1	0.23	0.32	45.63
#15	32.5	0.9	0.23	0.35	33.63
Extracts/molarity, µM	**THC**	**CBD**	**CBGA**	**CBN**	**TOTAL Cannabinoids**
#4	10.69	0.55	0.10	0.05	N/A
#6	3.28	7.44	1.07	0.03	N/A
#8	10.34	0.10	0.15	0.02	N/A
#12	13.74	0.57	0.29	0.04	N/A
#13	12.24	0.38	0.12	0.04	N/A
#14	14.09	0.35	0.07	0.10	N/A
#15	10.34	0.29	0.07	0.11	N/A

promising effects of novel cannabis extracts on the levels of key inflammation modulators in EpiDermFT model we further proceeded to substantiate our data and analyze the effects of extracts on lung fibroblasts. WI-38 cells were exposed to either TNFα-IFNγ alone or in combination with the indicated extracts for 48 h, and Western blotting was performed to determine the effect on COX2 and IL-6 expression. We noted that COX2 was induced by TNFα-IFNγ, this induction was attenuated by extracts #4, #6, #8, #12, and #15, while enhanced by #13 and #14 (Figure 4). Albeit TNFα-IFNγ had no effect on IL-6 induction, extracts #4, #6, and #8 downregulated, while extracts #13, #14, and #15 upregulated the levels of IL-6 in WI-38 cells (Figure 4).

DISCUSSION

Taken together, our results suggest that out of 7 studied extracts of novel *C. sativa* cultivars three were most effective down-regulating pro-inflammatory pathways and key cytokines implicated in the cytokine storm and ARDS in COVID-19. We noted that novel cannabis extracts down-regulated the levels of pro-inflammatory cytokine and interleukins, including IL-1 family, IL-23/IL-17 pathway, IL-6, IL-8, TNFα, and others that play key parts in inflammation and fibrosis. IL-1 family of interleukins is important in innate inflammation and autoimmunity [28]. IL-1α was shown to be constitutively present in numerous epithelial and mesenchymal cell types of healthy individuals, whereas

IL-1β is mainly induced under disease conditions [28]. Both pro-inflammatory interleukins are upregulated in numerous inflammatory and autoinflammatory diseases and are important druggable targets. Recent studies show that levels of IL-1 were strongly elevated in individuals with COVID-19, and IL-1 levels correlated with disease severity [29]. Increased expression of IL-23/IL-17 pathway was previously correlated with pulmonary inflammation in polymicrobial sepsis [30]. While on the one hand, the IL-17 family confers protection from a variety of extracellular pathogens and was shown to drive leukocyte infiltration to facilitate clearance of infectious pathogens, aberrant IL-17 signaling can lead to excess inflammation and tissue damage and fibrosis [31], and has been implicated in ARDS, cystic fibrosis, and pulmonary fibrosis and other pathological conditions (reviewed in [31]).

Together with interleukins and TNFα genes, novel cannabis extracts regulated the expression of various other genes involved in fibrosis, including pulmonary fibrosis (PF) (Table 1). Among those were metalloproteinases (MMPs), key proteases involved in ECM remodeling [23]. MMP1, MMP2, MMP7, and MMP9 were previously reported to be upregulated in PF.

CCL2, also known as MCP-1 (Figure 1 and Table 1), which is an important hallmark of fibrosis, and has been indicated as a potential druggable anti-fibrotic target [23]. In previous studies, CCL2 was shown to promote fibroblast differentiation and facilitate their recruitment to the alveolar space, thus leading to excessive collagen deposition [32]. Besides, CCL2 promoted fibroblast survival and stimulated IL-6 production [33]. Importantly, along with CCL2, IL-1, IL-6 and TNFα also regulate fibrosis [23], and their down-regulation may be viewed as a potential anti-fibrotic effect.

Overall, extracts down-regulated many pro-fibrotic genes, such as WNT5A, iCAM and others. Previous studies have shown that *in vivo* inhibition of WNT-5A attenuated tissue destruction, improved lung function and restoration of alveolar epithelial cell markers expression in two animal models of COPD [24, 34]. Down-regulation of iCAM1 and iCAM5 genes is also an important finding, as the levels of iCAM1 were shown to be elevated in sera of PF patients [35], and recent studies showed that iCAM-1 inhibition reduced exacerbations of lung inflammation [36].

Interestingly, in our study, extracts #6 and #13, along with down-regulation of numerous pro-inflammatory

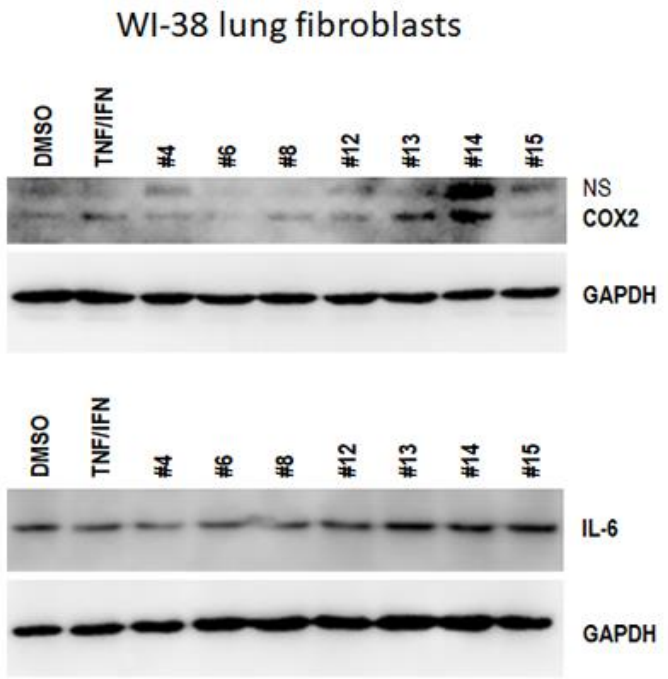

Figure 4. Effects of novel *C. sativa* extracts on the levels of IL-6 and COX2 in WI-38 lung fibroblasts. WI-38 cells grown to 80% confluency were treated with either 10 ng/ml TNFα /IFN γ alone or in combination with the indicated extracts; at 48 h after treatment, the whole cellular lysates were prepared and subjected to Western blot analysis using antibodies against IL-6 and COX2 as detailed in "Methods". GAPDH served as a loading control.

cytokines, upregulated CXCL12. The role of CXCL12 upregulation in PF still needs to be fully established, but, based on the current knowledge, CXCL12 upregulation can be viewed as a potential PF contributor, and thus its upregulation may negate the potential benefits of cytokine down-regulation by extracts #6 and #13.

Overall, pronounced inhibition of TNFα and IL-6 is the most important finding, as these molecules are currently considered to be the key actionable targets in COVID-19 cytokine storm and ARDS. Anti-cytokine therapies are thought to be important for prevention of COVID-19 pneumonia [37], as currently there is a race to develop novel anti-cytokine storm regimens. To that effect several anti-cytokine therapies have been proposed and are now in clinical trials. These include anti-IL-6 receptor antibody tocilizumab [11, 12, 38], colchicine, an agent that can potentially influence levels of IL-6 and other cytokines [39], chloroquine [15], metronidazole [40], and statins [41], as well as melatonin as an anti-inflammatory adjuvant therapy [6]. Chloroquine has some immunomodulatory effects, potentially suppressing the production and release of TNF-α and IL-6 [15]. Colchicine has been shown to effectively suppress interleukin IL-1b, IL-18 and IL-6 in patients with acute coronary syndrome [42, 43] and is now being trialed in COVID-19 ARDS, albeit it also has very significant side effects [39]. Nonetheless, a lot of studies yielded negative or controversial results, calling for more efforts aimed at the discovery of novel anti-cytokine storm regimens.

Several rheumatological drugs are now being evaluated for therapeutic potential to tame COVID-19 pneumonia, ARDS, and prevent further complications such as PF [29]. Suppression of pro-inflammatory IL-1 family members and IL-6 has been shown to have a therapeutic effect in many inflammatory diseases, including viral infections, and has been explored as a potential therapeutic avenue in COVID-19 [44]. A recent review summarized the roles of IL-6 in COVID-19 pathogenesis and highlighted the important therapeutic potential of the IL-6 blockade in COVID-19 management [45]. Interestingly, a recent in-depth meta-analysis of 1302 COVID-19 cases showed that the level of IL-6 was 3-fold higher in patients with severe vs mild/moderate COVID-19 ($p < 0.001$). Furthermore, high IL-6 levels correlated with the development of severe lung damage ($p = 0.001$) [11].

Numerous reports based on several observational or non-placebo controlled studies in patients with severe COVID-19 and ARDS suggest the therapeutic potential of these agents, especially tocilizumab, a monoclonal antibody against IL-6 receptor (reviewed in [45]).

Tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, is showing some promises, albeit it carries a hefty price tag and a lot of side effects [12, 46], and the results are not fully conclusive. Indeed, results of the COVIDOSE, low-dose tocilizumab in the treatment of Covid-19 trial, showed that low-dose tocilizumab administration led to rapid improvement in both laboratory and clinical manifestations of hyper-inflammation in patients hospitalized with COVID-19 [47]. On the other hand, preliminary results of COVACTA clinical trial reported no statistical difference between tocilizumab vs placebo arm in severe COVID-19 [45]. Currently several clinical trials are ongoing to ascertain the efficacy of tocilizumab - NCT04445272, NCT04479358 (COVIDOSE-2), NCT04317092 (TOCIVID), NCT04345445, including a phase 3 RCT (NCT04412772).

In parallel, a study reported successful treatment of COVID-19 pneumonia with clazakizumab, monoclonal antibody against human IL-6 [48]. Currently, five RCTs are ongoing to ascertain the therapeutic potential of anti-IL-6 antibody in COVID-19 (NCT04381052, NCT04348500, NCT04494724, NCT04343989, NCT04343989).

TNFα not only is the main cytokine storm driver, it also was shown to mediate the transition from pulmonary inflammation to fibrosis [49]. As a pro-inflammatory cytokine, TNFα is mechanistically involved in lung and vascular tissue damage, ARDS and coagulopathy [50, 51]. Elevated levels of TNFα and other pro-inflammatory cytokines and chemokines, such as IL-6, IL-10 are risk factors for the development of severe COVID-19, and their levels are much higher in critical patients than in those with milder disease [50, 52]. Furthermore, most recent *in vitro* data show that TNFα facilitates SARS-CoV-2 interaction with ACE2 receptor that is a key gateway of the virus into human cells [53]. Moreover, recent clinical case reports have shown that the use of anti-TNF therapies in patients with rheumatological conditions and mild cases of COVID-19 prevented their further progression to the severe COVID-19 forms, most probably by mitigating deleterious effects of the high levels of TNFα and other cytokines that drive immunopathogenesis of COVID-19 [51]. Surprisingly, up to now, no TNFα inhibitors have been trialed for COVID-19. The expert commentary in Lancet stated that "trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed" [54]. To that effect, a recent expert review identified opportunities for the use of TNFα inhibitors in COVID-19 [29]. The first phase 2 study aimed to evaluate whether or not early administration of TNFα inhibition by infliximab in patients with severe COVID-19 will reduce disease duration and severity (NCT04425538).

While potentially effective, anti-TNFα and anti-IL-6 and other anti-inflammatory biologics are very expensive and cause an array of side effects, including malignancies and their efficacy needs to be ascertained in clinical trials. On the other hand, anti-TNFα and anti-IL-6 cannabis extracts that are generally regarded as safe (GRAS) modalities can be a useful addition to the current anti-inflammatory regimens to treat COVID-19, as well as various rheumatological diseases and conditions such as rheumatoid arthritis (RA), psoriasis and psoriatic arthritis, osteoarthritis, fibromyalgia, and others. Indeed, cultivars targeting TNFα, IL-6, IL-1β and causing concerted and significant downregulation of the rheumatoid arthritis pathway, pending thorough verification and clinical validation, may present a novel and promising natural resource for RA treatments and management of other TNFα, IL-6, IL-1β-mediated diseases. Furthermore, a recent report shows that CBD and the combination of major terpenes was far superior than dexamethasone in treating COVID-19 [55], albeit a full and final report of this study is still pending.

While potentially important, our study has limitations. It was initially based on the use of human EpiDermFT 3D tissue model which is not the closest to the lung tissues. That being said, inflammation can be effectively induced in this model and it was curbed by the application of extracts. Our data need to be further substantiated using more lung cell lines and 3D tissue models of inflammation. Importantly, extracts that curbed inflammation is 3D tissues also exerted anti-inflammatory potential in WI-38 lung fibroblasts, albeit not at the same level. In the future, it would be important to further expand the study and include analysis of the effects in lung 3D tissues. Furthermore, recent screen of the battery of high-CBD extracts identified several that inhibited COX2 and other inflammation makers in lung tissues (data not shown). Notwithstanding, our study laid a foundation for the future analysis of the anti-inflammatory potency of cannabis and its applications for COVID-19 and ARDS.

CONCLUSIONS

Overall, we are the first to show that application of *C. sativa* extracts profoundly decreases the level of pro-inflammatory cytokines in human 3D tissues. Still, our study has several pitfalls. Here, we used human 3D full-thickness skin model to analyze the effects of cannabis extracts on inflammation and fibrosis. While it would be important to replicate the data in an airway epithelial and alveolar tissue models, and use either SARS-CoV2 virus or its components to induce inflammation, our data can be used as a roadmap for the future analysis. Moreover, key fundamental mechanisms of inflammation and fibrosis are similar in various tissues, and key roles of TNFα, IL-6 and other interleukins, chemokines, and MMPS have been well-established in an array of fibroproliferative diseases [5]. Pending further validation in lung tissue models, our novel extracts need to be studied in a clinical trial aimed to prevent or mitigate COVID-19 pneumonia and ARDS.

Most importantly, out of 7 selected extracts, only 3 performed best, one had no effects at all, and one exerted effects that may in turn appear to be deleterious, signifying that cannabis is not generic and careful cultivar selection must be based on thorough pre-clinical studies. Furthermore, the current study was developed to analyze the effects of medical cannabis applications rather than smoking.

In the future, anti-TNFα and anti-IL-6 extracts need to be analyzed for their potential to mitigate inflammation in rheumatoid arthritis, ankylosing spondylitis, and other rheumatologic conditions, especially given the fact that extracts profoundly downregulate the RA pathway and target TNFα and IL-6. Also, the effects of novel extracts also need to be analyzed for their potential to combat 'inflammaging' - the inflammatory underpinning of aging and frailty [56].

MATERIALS AND METHODS

Plant growth and extract preparation

All cannabis plants were grown in the licensed facility at the University of Lethbridge (license number LIC-62AHHG0R77-2019). *C. sativa* cultivars #4, #6, #8, #12, #13, #14, #15 were used for the experiments. Four plants per cultivar were grown at 22° C, 18 h light 6 h dark for 4 weeks and then transferred to the chambers with 12 h light/12 h dark regime to promote flowering. Plants were grown to maturity and flowers were harvested and dried. Flower samples from four plants per variety were combined and used for extraction. Three grams of the powdered plant tissue per each cultivar were used for extraction. Plant material was placed inside a 250 mL Erlenmeyer flask, 100 mL of ethyl acetate was poured into each flask. The flasks were covered with tin foil and incubated overnight in the dark at 21° C with continuous shaking at 120 rpm. Extracts were filtered, concentrated using a rotary vacuum evaporator and transferred to a tared 3-dram vial. The leftover solvent was evaporated to dryness in an oven overnight at 50° C to eliminate the solvent completely. Levels of cannabinoids was analysed using Agilent Technologies 1200 Series HPLC system. The extract stocks were prepared from the crude extracts, whereby 3-6 mg of crude extract was dissolved in DMSO (Dimethyl sulfoxide anhydrous, Life Technologies) to reach 60 mg/mL final concentration

and stored at -20° C. Appropriate cell culture media (RPMI + 10% FBS or EMEM + 10% FBS) were used to dilute the 60 mg/mL stock to make working medium containing 0.01 mg/ml. Extracts were sterilized using 0.22 µm filter.

Analysis of cannabinoids

Agilent Technologies 1200 Series HPLC system equipped with a G1315C DAD, G1316B column compartment, G1367D autosampler, and G1312B binary pump was used to analyse the acidic and neutral forms of phytocannabinoids. The separation was performed on a Phenomenex Kinetex EVO C18 column (5 µm, 100 x 2.1 mm id) with a Phenomenex SecurityGuard ULTRA guard column. Instrument control, data acquisition, and integration were done with ChemStation LC 3D Rev B.04.02 software (Agilent Technologies). A 2 µL injection volume was used for all calibration standards (THC, CBD, THC-A, CBD-A, CBG, CBG-A, all Sigma-Aldrich) and sample analysis. The compound peaks were detected for 230 nm and 280 nm. Mobile phases consisted of 50 mM ammonium formate (pH 5.19) (Sigma-Aldrich) in HPLC grade water (Fisher Chemical) on the A side and 100% methanol (Fisher Chemical) on the B side, with a flow rate 0.3 ml/min. Two samples per cultivar were analyzed, with two technical repeats per each sample. Data are presented in Table 3.

Analysis of terpenes

Terpene analysis was performed on dry flowers of cultivars #6, #8 and #12 using a 8610C GC coupled with a flame ionization detector (FID) from SRI Instruments at Canvas Labs (Vancouver, BC, Canada). Two samples per cultivar were analyzed.

Tissue and cell line models and treatments

Tissue models

EpiDermFTTM tissues were purchased from Mattek Life Sciences (Ashland, MA), equilibrated overnight under standard culture conditions (37° C, 5% CO2) with EpiDermFT Assay Media (EFT-400-ASY) and cultured according to manufacturer's instructions. Three tissues were used per extract. EpiDermFT recreates normal skin tissue structure with differentiated dermis and epidermis. It consists of human-derived epidermal keratinocytes and dermal fibroblasts that are mitotically and metabolically active. The tissues were cultured according to the manufacturer's protocol, using an air-liquid interface tissue culture technique.

To induce inflammation, tissues were exposed to UVC for 2 min, receiving 7000 erg. Distance from the light source was set to 10 cm. Upon exposure, tissues were treated with extracts. Specifically, right after the UVC treatment, the cannabis extracts (15 µl per sample) or vehicle (DMSO) were dissolved in media and applied to the media surrounding the tissues (n=3 for each condition). Control samples (PBS and DMSO) were sham treated – carried to the UVC source etc. but no UVC was given. The following experimental groups were set up:

"UNT" – untreated tissues;
"PBS" - 15 µl of 30% glycerol in PBS was applied to the tissues and no exposure was done;
"DMSO" - 15 µl of DMSO (0.017% in 30% glycerol-PBS) was applied to the tissues and no exposure was done;
"UV-PBS" - tissues were exposed to UV and 15 µl of 30% glycerol-PBS was applied to them;
"UV-DMSO" – tissues were exposed to UV and 15 µl of DMSO (0.017% in 30% glycerol-PBS) was applied to them;
"#4" through "#15" - tissues were exposed to UV and 15 µl of extracts in DMSO was applied to them.
Tissues were incubated with extracts for 24 h and flash frozen for RNA and protein analysis.

Cell line

WI-38 lung fibroblasts were purchased from the ATCC and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 fetal bovine serum according to the manufacturer's instructions. WI-38 cells grown to 80% confluency were treated with proinflammatory cytokines (10 ng/ml TNFα /IFN γ) alone or in combination with 0.015 µb/µL extracts, vehicle (DMSO) served as a control. At 48 h after treatment, the cells were washed twice with ice-cold PBS and lysed in a radioimmunoprecipitation assay buffer (RIPA).

Gene expression analysis

RNA isolation

Three tissues per group were used for the analysis of gene expression profiles. RNA was isolated from tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA), further purified using an RNAesy kit (Qiagen), and quantified using Nanodrop2000c (ThermoScientific). Afterwards, RNA integrity and concentration were determined using 2100 BioAnalyzer (Agilent).

Library construction and sequencing

In all cases, the sequencing libraries were prepared using NEBNext Ultra II mRNA library preparation kit for Illumina (NEB) following the manufacturer's instructions. The samples were processed by the same technician at the same time to avoid the introduction of

technical batch effects. The cDNA fragment libraries were sequenced using NextSeq500 sequencing analyzer (Illumina). The samples were balanced evenly across the lanes of the sequencing flowcell.

Bioinformatics analysis

Base-calling and demultiplexing were done with Illumina CASAVA v.1.9 bioinformatics pipeline. The base qualities were examined using FastQC v.0.11.8. The adapters and low-quality bases were trimmed using TrimGalore!v.0.6.4 https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/. Trimmed reads were mapped to the human genome version GRCh37 using HISAT2 version 2.0.5 [57]. Counts of reads mapping to the gene as a meta-feature were obtained using featureCounts v.1.6.1 [58] taking to account the directionality of the sequencing libraries. Counts of reads mapping to features were loaded into R v.3.6.1 and normalized using DESeq2 v.1.24.0 Bioconductor package as described in the manual [59]. The differences between all experimental groups were examined using the likelihood ratio test (LRT) test implemented in DESeq2. The reduced model included the intercept and the full model was the experimental group (Cannabis extracts and controls).

Pathway visualization was conducted using pathview v.1.26.0 Bioconductor package based on pathway schemes downloaded from Kyoto Encyclopedia of Genes and Genomes (KEGG) [60, 61]. Generally applicable gene set enrichment (GAGE) for pathway analysis method was used in unidirectional mode to detect experimentally perturbed KEGG pathways [62].

Statistics

Multiple comparisons adjustment of p-values was done using Benjamini-Hochberg procedure [63]. Specific comparisons between groups were extracted using *results()* function with *contrast* argument specified. Genes with adjusted p-values below 0.05 were considered significant.

Western blot analysis

After treatment with cannabis extracts for the indicated time, whole cellular lysates of 3D tissues were prepared in radioimmunoprecipitation assay buffer using 2.0 mm ZR BashingBead beads (Zymo Research). Proteins (30-100 µg per sample) were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Hybond™-P, GE Healthcare) at 4° C for 1.5 h. The blots were incubated for 1 h with 5% nonfat dry milk to block nonspecific binding sites and subsequently incubated at 4° C overnight with 1:1000 dilution of polyclonal antibody against IL-6 and COX-2 (Abcam). Immunoreactivity was detected using a peroxidase-conjugated antibody and visualized with the ECL Plus Western Blotting Detection System (GE Healthcare). The blots were stripped before reprobing with antibody against actin (Santa Cruz Biotechnology) or GAPDH (Abcam).

AUTHOR CONTRIBUTIONS

A.K., O.K. developed the idea; A.K. O.K. I.K, and B.W. planned the experiments; B.W., D.L., R. R-J. conducted experiments; A.K, S.Y., B.W, O.K. conducted initial data analysis; A.K, B.W, D.L., S.Y, I.K and O.K. conducted further data evaluation; A.K. B.W. and O.K. drafted the manuscript; all authors contributed to manuscript preparation and revision.

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CONFLICTS OF INTEREST

Pathway Rx is a startup company engaged in medical cannabis and disease research.

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REFERENCES

1. Kakodkar P, Kaka N, Baig MN. A comprehensive literature review on the clinical presentation, and management of the pandemic coronavirus disease 2019 (COVID-19). Cureus. 2020; 12:e7560. https://doi.org/10.7759/cureus.7560 PMID:32269893

2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

3. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Semin Immunopathol. 2017; 39:529–39. https://doi.org/10.1007/s00281-017-0629-x PMID:28466096

4. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LF. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol. 2020; 20:363–74. https://doi.org/10.1038/s41577-020-0311-8 PMID:32346093

5. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Invest. 2007; 117:524–29. https://doi.org/10.1172/JCI31487 PMID:17332879

6. Zhang R, Wang X, Ni L, Di X, Ma B, Niu S, Liu C, Reiter RJ. COVID-19: melatonin as a potential adjuvant treatment. Life Sci. 2020; 250:117583. https://doi.org/10.1016/j.lfs.2020.117583 PMID:32217117

7. Pedersen SF, Ho YC. SARS-CoV-2: a storm is raging. J Clin Invest. 2020; 130:2202–05. https://doi.org/10.1172/JCI137647 PMID:32217834

8. Guo YR, Cao QD, Hong ZS, Tan YY, Chen SD, Jin HJ, Tan KS, Wang DY, Yan Y. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak - an update on the status. Mil Med Res. 2020; 7:11. https://doi.org/10.1186/s40779-020-00240-0 PMID:32169119

9. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

10. Liu Q, Zhou YH, Yang ZQ. The cytokine storm of severe influenza and development of immunomodulatory therapy. Cell Mol Immunol. 2016; 13:3–10. https://doi.org/10.1038/cmi.2015.74 PMID:26189369

11. Coomes EA, Haghbayan H. Interleukin-6 in covid-19: a systematic review and meta-analysis. Rev Med Virol. 2020; 30:1–9. https://doi.org/10.1002/rmv.2141 PMID:32845568

12. Buonaguro FM, Puzanov I, Ascierto PA. anti-IL6R role in treatment of COVID-19-related ARDS. J Transl Med. 2020; 18:165. https://doi.org/10.1186/s12967-020-02333-9 PMID:32290847

13. Radner H, Aletaha D. anti-TNF in rheumatoid arthritis: an overview. Wien Med Wochenschr. 2015; 165:3–9. https://doi.org/10.1007/s10354-015-0344-y PMID:25651945

14. Tanaka T, Narazaki M, Kishimoto T. Immunotherapeutic implications of IL-6 blockade for cytokine storm. Immunotherapy. 2016; 8:959–70. https://doi.org/10.2217/imt-2016-0020 PMID:27381687

15. Huang KJ, Su IJ, Theron M, Wu YC, Lai SK, Liu CC, Lei HY. An interferon-gamma-related cytokine storm in SARS patients. J Med Virol. 2005; 75:185–94. https://doi.org/10.1002/jmv.20255 PMID:15602737

16. Chen JY, Qiao K, Liu F, Wu B, Xu X, Jiao GQ, Lu RG, Li HX, Zhao J, Huang J, Yang Y, Lu XJ, Li JS, et al. Lung transplantation as therapeutic option in acute respiratory distress syndrome for coronavirus disease 2019-related pulmonary fibrosis. Chin Med J (Engl). 2020; 133:1390–96. https://doi.org/10.1097/CM9.0000000000000839 PMID:32251003

17. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008; 214:199–210. https://doi.org/10.1002/path.2277 PMID:18161745

18. Tang C, Chen S, Qian H, Huang W. Interleukin-23: as a drug target for autoimmune inflammatory diseases. Immunology. 2012; 135:112–24. https://doi.org/10.1111/j.1365-2567.2011.03522.x PMID:22044352

19. Robert M, Miossec P. IL-17 in rheumatoid arthritis and precision medicine: from synovitis expression to circulating bioactive levels. Front Med (Lausanne). 2019; 5:364. https://doi.org/10.3389/fmed.2018.00364 PMID:30693283

20. Mistry P, Laird MH, Schwarz RS, Greene S, Dyson T, Snyder GA, Xiao TS, Chauhan J, Fletcher S, Toshchakov VY, MacKerell AD Jr, Vogel SN. Inhibition of TLR2 signaling by small molecule inhibitors targeting a pocket within the TLR2 TIR domain. Proc Natl Acad Sci USA. 2015; 112:5455–60. https://doi.org/10.1073/pnas.1422576112 PMID:25870276

21. Arora S, Ahmad S, Irshad R, Goyal Y, Rafat S, Siddiqui N, Dev K, Husain M, Ali S, Mohan A, Syed MA. TLRs in pulmonary diseases. Life Sci. 2019; 233:116671. https://doi.org/10.1016/j.lfs.2019.116671 PMID:31336122

22. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol. 2009; 1:a001651. https://doi.org/10.1101/cshperspect.a001651 PMID:20457564

23. Kolahian S, Fernandez IE, Eickelberg O, Hartl D. Immune mechanisms in pulmonary fibrosis. Am J Respir Cell Mol Biol. 2016; 55:309–22. https://doi.org/10.1165/rcmb.2016-0121TR PMID:27149613

24. Baarsma HA, Königshoff M. 'WNT-er is coming': Wnt signalling in chronic lung diseases. Thorax. 2017; 72:746–59. https://doi.org/10.1136/thoraxjnl-2016-209753 PMID:28416592

25. Königshoff M, Balsara N, Pfaff EM, Kramer M, Chrobak I, Seeger W, Eickelberg O. Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One. 2008; 3:e2142. https://doi.org/10.1371/journal.pone.0002142 PMID:18478089

26. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest. 2004; 114:438–46. https://doi.org/10.1172/JCI20997 PMID:15286810

27. Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol. 2011; 163:1344–64. https://doi.org/10.1111/j.1476-5381.2011.01238.x PMID:21749363

28. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. Immunol Rev. 2018; 281:8–27. https://doi.org/10.1111/imr.12621 PMID:29247995

29. Perricone C, Triggianese P, Bartoloni E, Cafaro G, Bonifacio AF, Bursi R, Perricone R, Gerli R. The anti-viral facet of anti-rheumatic drugs: lessons from COVID-19. J Autoimmun. 2020; 111:102468. https://doi.org/10.1016/j.jaut.2020.102468 PMID:32317220

30. Cauvi DM, Williams MR, Bermudez JA, Armijo G, De Maio A. Elevated expression of IL-23/IL-17 pathway-related mediators correlates with exacerbation of pulmonary inflammation during polymicrobial sepsis. Shock. 2014; 42:246–55. https://doi.org/10.1097/SHK.0000000000000207 PMID:24978886

31. Gurczynski SJ, Moore BB. IL-17 in the lung: the good, the bad, and the ugly. Am J Physiol Lung Cell Mol Physiol. 2018; 314:L6–16. https://doi.org/10.1152/ajplung.00344.2017 PMID:28860146

32. Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am J Pathol. 2005; 166:675–84. https://doi.org/10.1016/S0002-9440(10)62289-4 PMID:15743780

33. Liu X, Das AM, Seideman J, Griswold D, Afuh CN, Kobayashi T, Abe S, Fang Q, Hashimoto M, Kim H, Wang X, Shen L, Kawasaki S, Rennard SI. The CC chemokine ligand 2 (CCL2) mediates fibroblast survival through IL-6. Am J Respir Cell Mol Biol. 2007; 37:121–28. https://doi.org/10.1165/rcmb.2005-0253OC PMID:17379849

34. Lam AP, Herazo-Maya JD, Sennello JA, Flozak AS, Russell S, Mutlu GM, Budinger GR, DasGupta R, Varga J, Kaminski N, Gottardi CJ. Wnt coreceptor Lrp5 is a driver of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2014; 190:185–95. https://doi.org/10.1164/rccm.201401-0079OC PMID:24921217

35. Tsoutsou PG, Gourgoulianis KI, Petinaki E, Mpaka M, Efremidou S, Maniatis A, Molyvdas PA. ICAM-1, ICAM-2 and ICAM-3 in the sera of patients with idiopathic pulmonary fibrosis. Inflammation. 2004; 28:359–64. https://doi.org/10.1007/s10753-004-6647-6 PMID:16245079

36. Traub S, Nikonova A, Carruthers A, Dunmore R, Vousden KA, Gogsadze L, Hao W, Zhu Q, Bernard K, Zhu J, Dymond M, McLean GR, Walton RP, et al. An anti-human ICAM-1 antibody inhibits rhinovirus-induced exacerbations of lung inflammation. PLoS Pathog. 2013; 9:e1003520. https://doi.org/10.1371/journal.ppat.1003520 PMID:23935498

37. Monteleone G, Sarzi-Puttini PC, Ardizzone S. Preventing COVID-19-induced pneumonia with anticytokine therapy. Lancet Rheumatol. 2020; 2:e255–56. https://doi.org/10.1016/S2665-9913(20)30092-8 PMID:32368737

38. Fu B, Xu X, Wei H. Why tocilizumab could be an effective treatment for severe COVID-19? J Transl Med. 2020; 18:164. https://doi.org/10.1186/s12967-020-02339-3 PMID:32290839

39. Deftereos SG, Siasos G, Giannopoulos G, Vrachatis DA, Angelidis C, Giotaki SG, Gargalianos P, Giamarellou H, Gogos C, Daikos G, Lazanas M, Lagiou P, Saroglou G, et al. The greek study in the effects of colchicine in COvid-19 complications prevention (GRECCO-19 study): rationale and study design. Hellenic J Cardiol. 2020; 61:42–45. https://doi.org/10.1016/j.hjc.2020.03.002 PMID:32251729

40. Gharebaghi R, Heidary F, Moradi M, Parvizi M. Metronidazole; a potential novel addition to the COVID-19 treatment regimen. Arch Acad Emerg Med. 2020; 8:e40. PMID:32259129

41. Dashti-Khavidaki S, Khalili H. Considerations for Statin Therapy in Patients with COVID-19. Pharmacotherapy. 2020; 40:484–486. https://doi.org/10.1002/phar.2397 PMID:32267560

42. Martínez GJ, Robertson S, Barraclough J, Xia Q, Mallat Z, Bursill C, Celermajer DS, Patel S. Colchicine acutely suppresses local cardiac production of inflammatory cytokines in patients with an acute coronary syndrome. J Am Heart Assoc. 2015; 4:e002128. https://doi.org/10.1161/JAHA.115.002128 PMID:26304941

43. Robertson S, Martínez GJ, Payet CA, Barraclough JY, Celermajer DS, Bursill C, Patel S. Colchicine therapy in acute coronary syndrome patients acts on caspase-1 to suppress NLRP3 inflammasome monocyte activation. Clin Sci (Lond). 2016; 130:1237–46. https://doi.org/10.1042/CS20160090 PMID:27129183

44. Conti P, Ronconi G, Caraffa A, Gallenga CE, Ross R, Frydas I, Kritas SK. Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. J Biol Regul Homeost Agents. 2020. https://doi.org/10.23812/CONTI-E PMID:32171193

45. Nasonov E, Samsonov M. The role of interleukin 6 inhibitors in therapy of severe COVID-19. Biomed Pharmacother. 2020; 131:110698. https://doi.org/10.1016/j.biopha.2020.110698 PMID:32920514

46. Zhang C, Wu Z, Li JW, Zhao H, Wang GQ. Cytokine release syndrome in severe COVID-19: interleukin-6 receptor antagonist tocilizumab may be the key to reduce mortality. Int J Antimicrob Agents. 2020; 55:105954. https://doi.org/10.1016/j.ijantimicag.2020.105954 PMID:32234467

47. Strohbehn GW, Heiss BL, Rouhani SJ, Trujillo JA, Yu J, Kacew AJ, Higgs EF, Bloodworth JC, Cabanov A, Wright RC, Koziol A, Weiss A, Danahey K, et al. COVIDOSE: low-dose tocilizumab in the treatment of covid-19. medRxiv. 2020; 2020:07.20. https://doi.org/10.1101/2020.07.20.20157503 PMID:32743594

48. Vaidya G, Czer LS, Kobashigawa J, Kittleson M, Patel J, Chang D, Kransdorf E, Shikhare A, Tran H, Vo A, Ammerman N, Huang E, Zabner R, Jordan S. Successful treatment of severe COVID-19 pneumonia with clazakizumab in a heart transplant recipient: a case report. Transplant Proc. 2020; 52:2711–14. https://doi.org/10.1016/j.transproceed.2020.06.003 PMID:32563584

49. Oikonomou N, Harokopos V, Zalevsky J, Valavanis C, Kotanidou A, Szymkowski DE, Kollias G, Aidinis V. Soluble TNF mediates the transition from pulmonary inflammation to fibrosis. PLoS One. 2006; 1:e108. https://doi.org/10.1371/journal.pone.0000108 PMID:17205112

50. Li G, Fan Y, Lai Y, Han T, Li Z, Zhou P, Pan P, Wang W, Hu D, Liu X, Zhang Q, Wu J. Coronavirus infections and immune responses. J Med Virol. 2020; 92:424–32. https://doi.org/10.1002/jmv.25685 PMID:31981224

51. Brito CA, Paiva JG, Pimentel FN, Guimarães RS, Moreira MR. COVID-19 in patients with rheumatological diseases treated with anti-TNF. Ann Rheum Dis. 2020. [Epub ahead of print]. https://doi.org/10.1136/annrheumdis-2020-218171 PMID:32546603

52. Fazeli PK, Lee H, Steinhauser ML. Aging is a powerful risk factor for type 2 diabetes mellitus independent of body mass index. Gerontology. 2020; 66:209–10. https://doi.org/10.1159/000501745 PMID:31505500

53. Haga S, Yamamoto N, Nakai-Murakami C, Osawa Y, Tokunaga K, Sata T, Yamamoto N, Sasazuki T, Ishizaka Y. Modulation of TNF-alpha-converting enzyme by the spike protein of SARS-CoV and ACE2 induces TNF-alpha production and facilitates viral entry. Proc Natl Acad Sci USA. 2008; 105:7809–14. https://doi.org/10.1073/pnas.0711241105 PMID:18490652

54. Feldmann M, Maini RN, Woody JN, Holgate ST, Winter G, Rowland M, Richards D, Hussell T. Trials of anti-tumour necrosis factor therapy for COVID-19 are urgently needed. Lancet. 2020; 395:1407–09. https://doi.org/10.1016/S0140-6736(20)30858-8 PMID:32278362

55. Earlenbaugh E. New Research Suggests Terpenes And CBD Work 2X's Better For Covid-19 Inflammation Than Corticosteroid. Forbes. 2020, 2020.

56. Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: a new immune-metabolic viewpoint for age-related diseases. Nat Rev Endocrinol. 2018; 14:576–90. https://doi.org/10.1038/s41574-018-0059-4 PMID:30046148

57. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015; 12:357–60. https://doi.org/10.1038/nmeth.3317 PMID:25751142

58. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014; 30:923–30. https://doi.org/10.1093/bioinformatics/btt656 PMID:24227677

59. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15:550. https://doi.org/10.1186/s13059-014-0550-8 PMID:25516281

60. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. Bioinformatics. 2013; 29:1830–31. https://doi.org/10.1093/bioinformatics/btt285 PMID:23740750

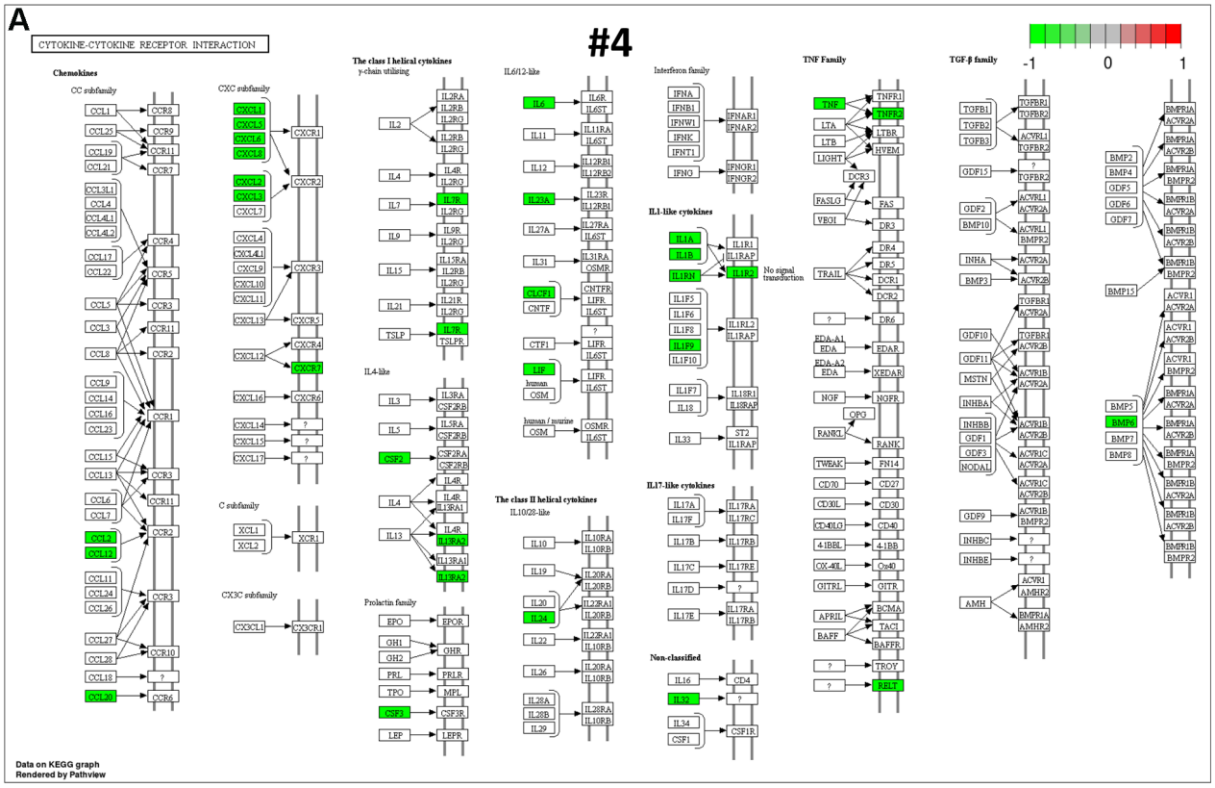
61. Wixon J, Kell D. The kyoto encyclopedia of genes and genomes—KEGG. Yeast. 2000; 17:48–55. https://doi.org/10.1002/(SICI)1097-0061(200004)17:1<48::AID-YEA2>3.0.CO;2-H PMID:10928937

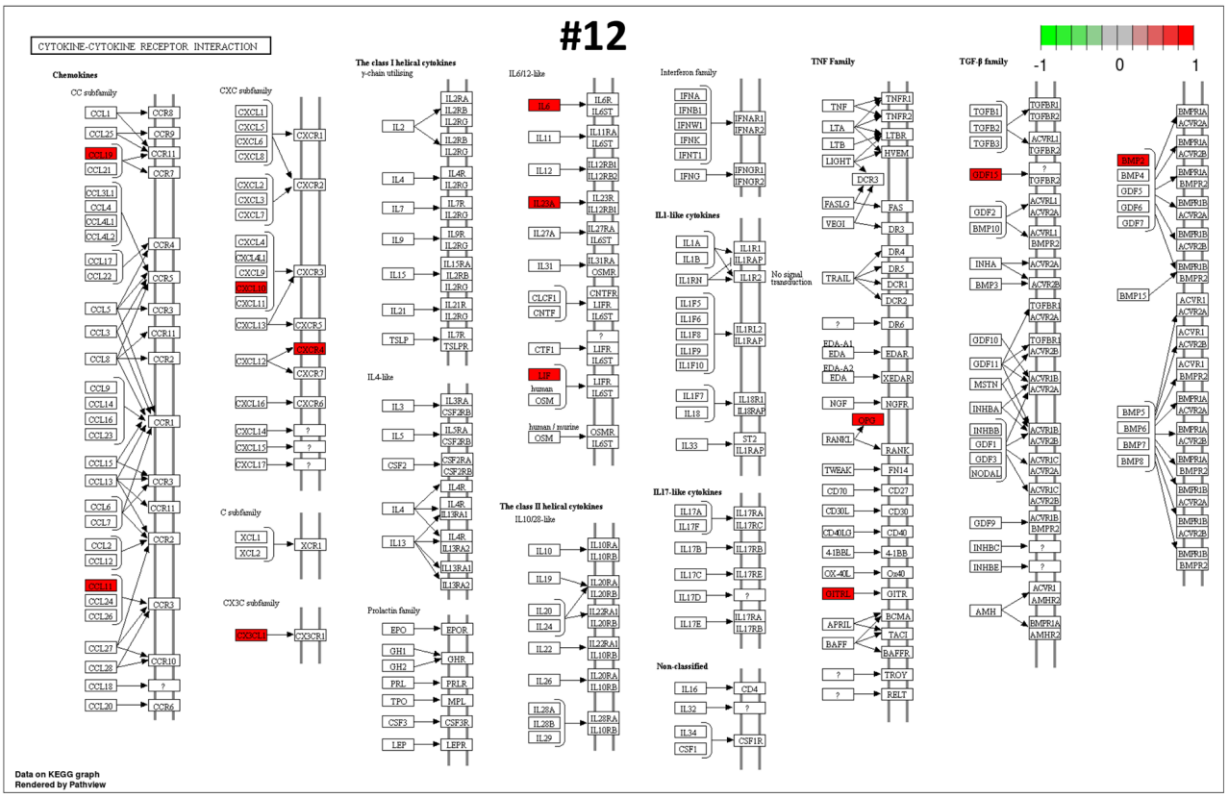
62. Luo W, Friedman MS, Shedden K, Hankenson KD, Woolf PJ. GAGE: generally applicable gene set enrichment for pathway analysis. BMC Bioinformatics. 2009; 10:161. https://doi.org/10.1186/1471-2105-10-161 PMID:19473525

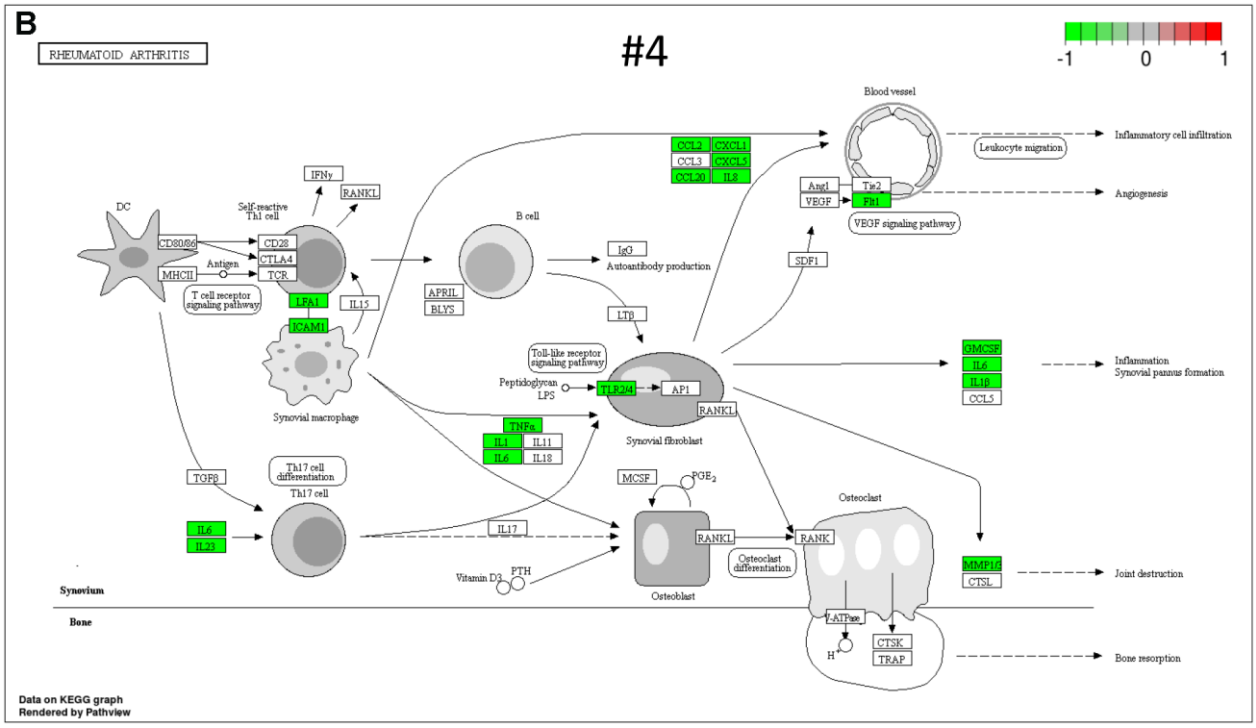
63. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological) 1995; 57:289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

SUPPLEMENTARY MATERIALS

Supplementary Figure



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T cell receptor signaling pathway
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ICAM1
Synovial macrophage
B cell
IgG
Autoantibody production
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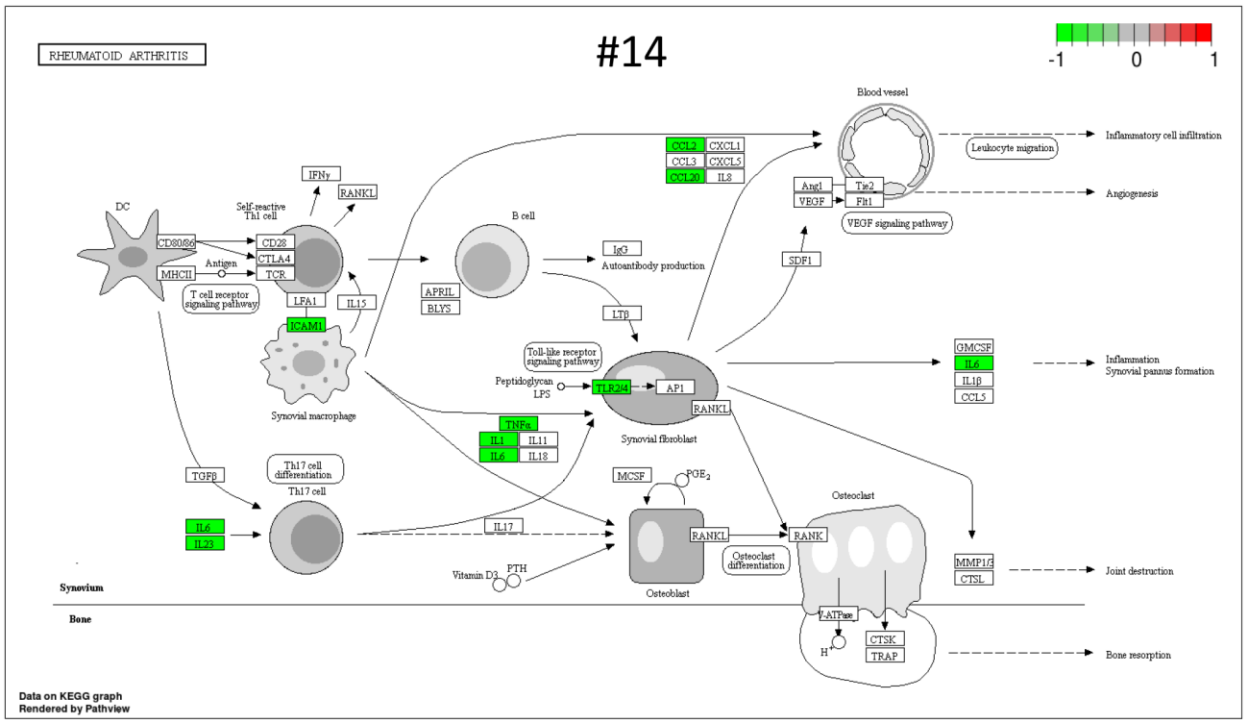
Data on KEGG graph
Rendered by Pathview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CD28
CTLA4
TCR
MHCII
Antigen
T cell receptor signaling pathway
LFA1
IL15
ICAM1
Synovial macrophage
B cell
IgG
Autoantibody production
APRIL
BLYS
LTβ
SDF1
CCL2
CXCL1
CCL3
CXCL5
CCL20
IL8
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VEGF
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Toll-like receptor signaling pathway
Peptidoglycan
LPS
TLR2/4
AP1
RANKL
Synovial fibroblast
GMCSF
IL6
IL1β
CCL5
Inflammation
Synovial pannus formation
TNFα
IL1
IL11
IL6
IL18
TGFβ
Th17 cell differentiation
Th17 cell
IL6
IL23
IL17
MCSF
PGE2
Osteoclast
RANK
RANKL
Osteoclast differentiation
Vitamin D3
PTH
Osteoblast
MMP1/3
CTSL
Joint destruction
V-ATPase
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CTSK
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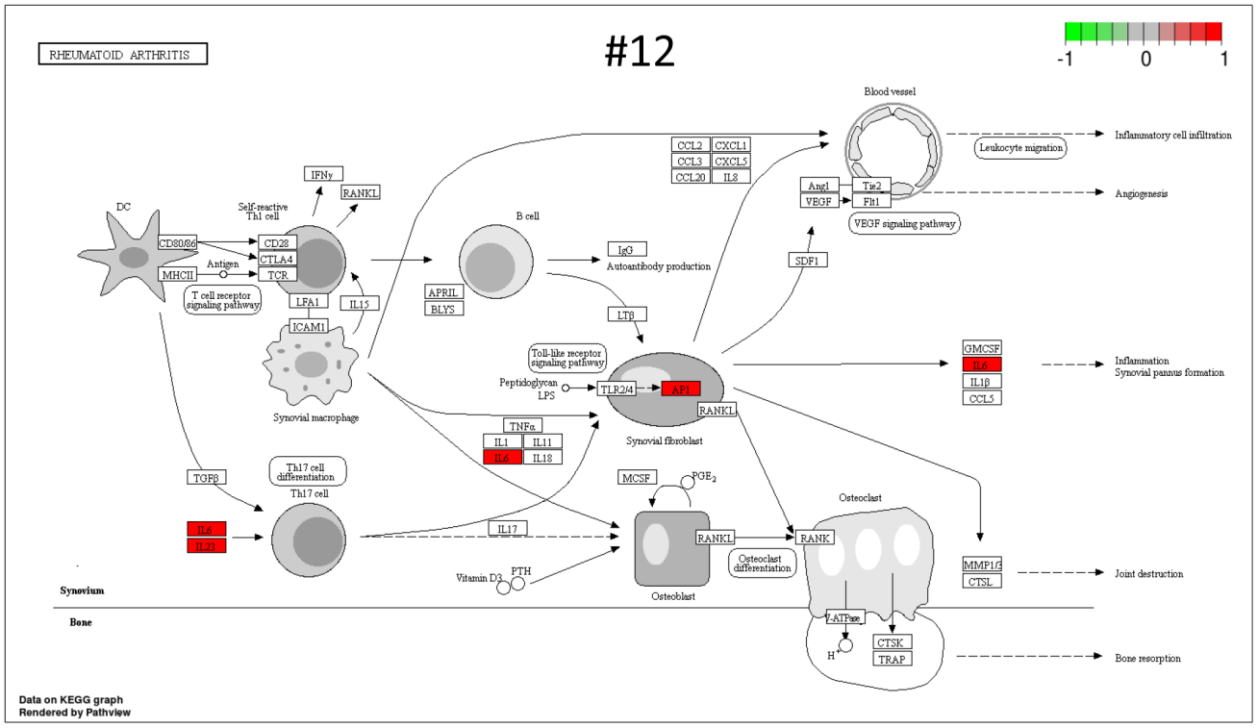
Bone resorption
Synovium
Bone
Data on KEGG graph
Rendered by Pathview



Supplementary Figure 1. Effects of selected extracts on 3D tissues on cytokine-cytokine receptor interactions and rheumatoid arthritis pathways. Generally applicable gene set enrichment (GAGE) for pathway analysis method was used in unidirectional mode to detect experimentally perturbed KEGG pathways [62]. (**A**) Changes in the cytokine-cytokine receptor interactions pathway caused by extracts #4 and #12. (**B**) Changes in the rheumatoid arthritis pathway caused by extracts #4, #14 and #12. Red – upregulation; green – down-regulation.

Review

Neurological involvement in the respiratory manifestations of COVID-19 patients

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ABSTRACT

The peculiar features of coronavirus disease 2019 (COVID-19), caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), are challenging the current biological knowledge. Early in Feb, 2020, we suggested that SARS-CoV-2 may possess neuroinvasive potential similar to that of many other coronaviruses. Since then, a variety of neurological manifestations have been associated with SARS-CoV-2 infection, which was supported in some patients with neuroimaging and/or cerebrospinal fluid tests. To date, at least 27 autopsy studies on the brains of COVID-19 patients can be retrieved through PubMed/MEDLINE, among which neuropathological alterations were observed in the brainstem in 78 of 134 examined patients, and SARS-CoV-2 nucleic acid and viral proteins were detected in the brainstem in 16/49 (32.7%) and 18/71 (25.3%) cases, respectively. To shed some light on the peculiar respiratory manifestations of COVID-19 patients, this review assessed the existing evidence about the neurogenic mechanism underlying the respiratory failure induced by SARS-CoV-2 infection. Acknowledging the neurological involvement has important guiding significance for the prevention, treatment, and prognosis of SARS-CoV-2 infection.

INTRODUCTION

Since the beginning of 2020, a newly emerging coronavirus (CoV), known as Severe Acute Respiratory Syndrome CoV-2 (SARS-CoV-2), has spread rapidly among human beings all over the world, leading to a disease called coronavirus disease 2019 (COVID-19). SARS-CoV-2 not only causes acute, highly lethal pneumonia, but also infects many other systems, including the immune, cardiovascular, digestive, urinary, and nervous systems.

As of Jan 2020, only 24 articles on COVID-19 can be found through PubMed/MEDLINE, however, the number of papers increased exponentially over the next few months (Figure 1A). By Oct 31, 2020, over 64,000 articles on COVID-19 can be retrieved, indicating that the pandemic of COVID-19 has aroused great public concerns. Among the published data, 57.7% are related to organ involvement. The papers on the respiratory, immune, cardiovascular, digestive, urinary and nerve systems account for 28.6%, 9.4%, 5.0%, 4.5%, 3.2% and 3.0%, respectively. The remaining 42.3% focus mainly on disease prevention and treatment, virus structure, vaccines, epidemiological characteristics and so on (Figure 1B).

To provide a clue for the prevention, treatment, or further study of COVID-19, we suggested early in Feb 2020 that SARS-CoV-2 may have similar neuroinvasive potential to that of many other CoVs [1, 2]. In Feb 2020, only two articles can be found on the neurology or neuroscience of COVID-19. However, by Oct 31, 2020, more than 1900 articles on this topic can be

retrieved, most of which were published after Apr 2020 (Figure 1C).

Neurological involvement in COVID-19, now called "COVID-19 neuroscience" or "COVID-19 neurology", has attracted more and more attention [3, 4]. Elucidating the underlying mechanisms assist in formulating effective treatment strategies to reduce the mortality of SARS-CoV-2 infection. In this paper, we review the existing evidence regarding SARS-CoV-2 neuroinvasion and further explore its possible implications in the respiratory manifestations of COVID-19 patients.

Evidence for the neuroinvasion of SARS-CoV-2

To date, a variety of neurological manifestations have been documented after SARS-CoV-2 infection. Neurological involvement in some patients were supported by neuroimaging findings [5–8] and positive detection of SARS-CoV-2 RNA in cerebrospinal fluid (CSF) [9–30]. Moreover, SARS-CoV-2 RNA and/or viral proteins were detected in the brains of some patients who died from COVID-19 [31–37].

The first-hand clinical report on neurological manifestations associated with SARS-CoV-2 infection was available as a preprint in medRxiv early in Feb, 2020, which was then published in *JAMA* in Apr, 2020 [5]. According to this report, 36.4% of the patients presented various neurological manifestations. Thereafter, Romero-Sánchez et al. evaluated 841 COVID-19 patients in Spain and found that 57.4% exhibited various neurological symptoms [6]. Pinna et al. analyzed the clinical records of 650 COVID-19 patients in Chicago, USA, and found that 7.7% of the patients showed neurological symptoms [7]. Similarly, Karadaş et al. evaluated 239 consecutive inpatients with COVID-19 in Ankara, Turkey, and detected neurological symptoms in 83 (34.7%) patients [8].

The COVID-19-associated neurological symptoms can be classified into three categories: 1) central nervous system (CNS) involvement, including headache, dizziness, consciousness disorder, epilepsy, and acute cerebrovascular accidents; 2) peripheral nervous system (PNS) involvement, such as olfactory loss, hypogeusia, visual impairment, and neuralgia, and 3) skeletal muscle injury [38]. Agarwal et al. analyzed the clinical data of 404 patients with COVID-19 in Washington, USA [39], and found that the most common CNS involvement was impaired consciousness (21.3%), followed by headache (20.3%) and dizziness (7.7%). The most common PNS involvement was muscle pain (32.4%), followed by the disorders of taste (6.7%) and smell (4.5%). Approximately 24.5% of COVID-19 patients showed acute neurological symptoms, of which the most

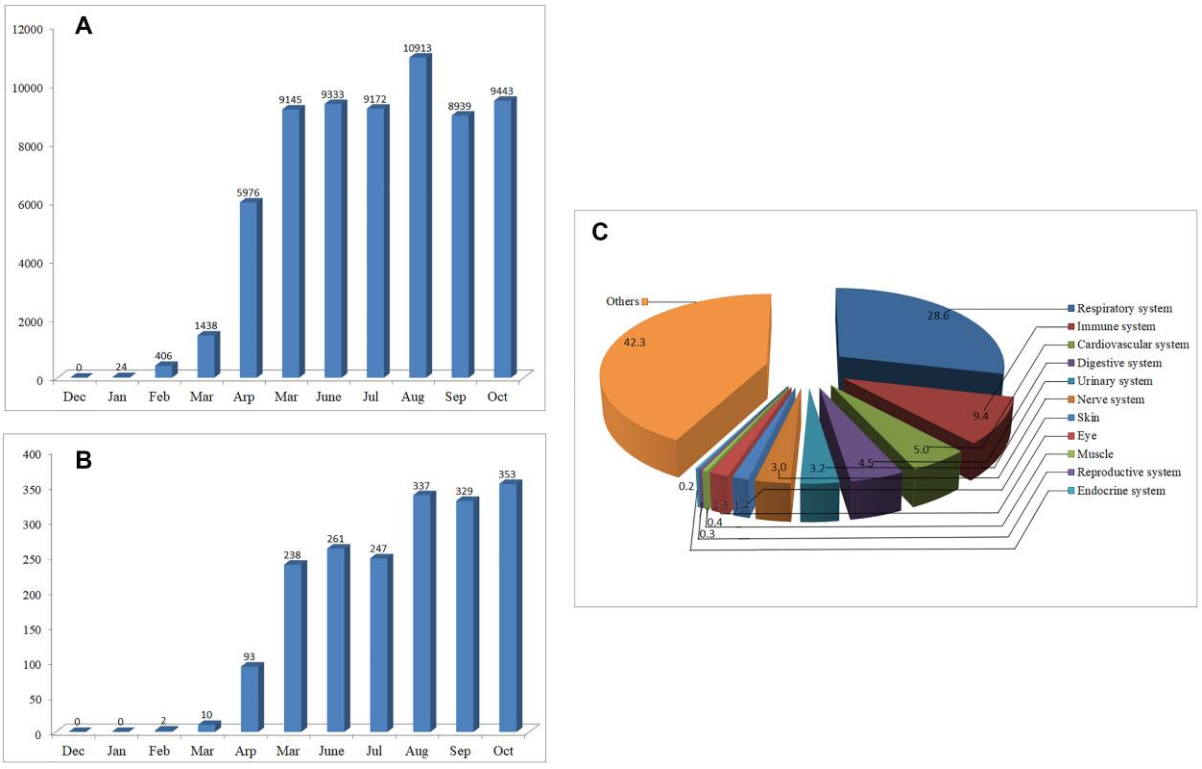

Figure 1. The number of articles on COVID-19 published from Dec 01, 2019 to Oct 31, 2020. (**A**) Monthly changes of the number of articles on COVID-19. (**B**) Monthly changes of the number of articles on COVID-19 neurology. (**C**) Percentage of the published articles on different systems of the body.

common was mental state changes (21.3%), followed by critical illness myopathy (2.0%), stroke (0.7%), and seizures (0.5%) [39].

Of interest, neurological manifestations were reported to be the initial or the only symptom in many patients with COVID-19 [13, 24, 40–42], indicating that the nervous system may be one of the primary targets of SARS-CoV-2. Many neurological manifestations, especially those reported in critical patients with COVID-19, may be attributed to systemic inflammatory responses, hypoxemia, or multi-organ failure [43]. However, some special neurological manifestations, such as encephalitis, anosmia and hyposmia, may be related to the direct invasion of SARS-CoV-2 into the CNS.

Since Moriguchi et al. [24] and Xiang et al. [30] provided the first evidence of SARS-CoV-2 in the CSF of COVID-19 patients, so far, at least 26 cases have been reported to show positive CSF detection of SARS-CoV-2 [9–30]. Interestingly, in some cases SARS-CoV-2 RNA was detected in the neural tissues, but not in the CSF [31, 36]. Since the CSF test is related to the time of CSF collection, the severity of infection, or the sensitivity of detection methods, the negative outcomes of CSF tests are not equated with the absence of SARS-CoV-2 in the CNS [43–45].

Previous studies on some other neurotropic viruses show that invasion of viruses into the CNS is associated with the increase of intrathecal antibodies in the CSF [46–47]. Song et al. analyzed CSF samples from 6 COVID-19 patients, including 3 with encephalopathy, 2 with intractable headache, and 1 with seizures [48]. Strikingly, antibodies specific for SARS-CoV-2 were observed in the CSF in all patients. Using an animal model expressing human angiotensin-converting enzyme 2 (ACE2), they further found that the antibodies appeared or increased in the CSF only when the CNS was infected. As a matter of fact, antibodies specific for SARS-CoV-2 have been found in the CSF in 30 patients with COVID-19 in 6 case/case series reports [26, 49–53]. According to the results reported by Song et al. [48], the anti-SARS-CoV-2 antibodies in the CSF in patients with intact blood-brain barrier are closely related to the direct invasion of the virus into the CNS.

In support of SARS-CoV-2 neuroinvasion, Paniz-Mondolfi et al. reported the first autopsy evidence of the presence of SARS-CoV-2 in the brain of one COVID-19 patient on Apr 21, 2020 [31]. Since then, more and more autopsy studies show that SARS-CoV-2 can enter the CNS and infect a variety of brain regions [32–37]. To date, SARS-CoV-2 RNA and/or viral proteins have been detected in the olfactory mucosa/nerve/bulb [32–33], trigeminal ganglion [32–33], medulla oblongata [32, 37], cerebrum [35], and cerebellum [36].

Consistent with the hypoxemic and hypercoagulable state in most decreased patients, cerebrovascular accidents [35, 48, 54–59] and/or hypoxic lesions [54, 56, 58, 60–62] have been widely observed in the brain in COVID-19 patients. However, these are not contradictory to the neuroinvasion of SARS-CoV-2, since SARS-CoV-2 RNA and/or viral proteins were detected in the brains of patients with cerebrovascular diseases and/or hypoxic injury [37, 48, 54, 56, 59–60]. In addition, many autopsy studies observed severe microgliosis and/or lymphocytic infiltration in specific brain regions [35, 54, 58], especially in the brainstem [37, 54, 56–57, 62–63].

The extensive presence of SARS-CoV-2 in the CNS, as well as the distinctly different neuropathological changes, is well consistent with the broad spectrum of neurological dysfunctions documented in patients with COVID-19.

The neuroinvasion of SARS-CoV-2 is associated with respiratory manifestations in COVID-19 patients

Respiratory failure is a major cause of high mortality induced by SARS-CoV-2 infection [64]. Approximately 10% of COVID-19 patients who developed respiratory failure had to be transferred to intensive care unit (ICU) for ventilatory support, and up to 79% of them died [65–66]. Therefore, clarifying the underlying mechanism is urgently needed to make a reasonable treatment plan to save patients' lives.

Based on the clinical and experimental data available for CoVs, we previously suggested that the neuroinvasive potential of SARS-CoV-2 may play a role in the acute respiratory failure of some COVID-19 patients [1, 2]. In this section, we discuss the peculiar respiratory manifestations of COVID-19 patients and further assess the existing evidence of the neurological involvement in the respiratory failure induced by SARS-CoV-2 infection.

Lung injury alone cannot explain the respiratory performance of all the patients with COVID-19

Radiographic studies show that most hospitalized patients with COVID-19 showed bilateral multiple peripheral ground-glass opacities at chest computerized tomography (CT) examination [65, 67]. The development of lung lesions on chest CT is generally consistent with the clinical time course of COVID-19 progression [68].

According to a study by Wang et al., the extent of lung lesions was similar in all 138 COVID-19 patients, whether mild or severe [69]. This finding was further confirmed by several other studies [65, 70–71]. Surprisingly, asymptomatic patients were reported to show similar imaging abnormalities without a significant difference from those in symptomatic patients [72].

Despite obvious lung abnormalities, most COVID-19 patients showed only mild flu-like symptoms. Approximately 37.8 ~ 67.8% of patients presented cough [73–74, 71], but most of them did not have sputum production. The productive cough was present only in 12.1 ~ 35.9% of mild patients and 22.2 ~ 48.8% of severe patients [69, 73–75]. However, hypoxemia may develop in both mild and severe patients with COVID-19 [70, 76–77]. Of interest, 29.4 ~ 62.4% of severe patients and 74.4 ~ 84.9% of mild patients did not present dyspnea [73–75].

Many patients with SARS-CoV-2 infection came to hospitals with severe hypoxemia so that they should have lost consciousness or be close to organ failure. Surprisingly, they denied any difficulty with breathing, and showed no signs of using auxiliary respiratory muscles. This unusual clinical presentation has been termed as "silent hypoxia", and is defying the current basic biology [78]. In some COVID-19 patients, the "silent" hypoxemia might last for a long time after receiving symptomatic support treatment, which gave medical staff an illusion of improvement. However, hypoxemia in some cases suddenly progressed and worsened from 10 ~ 14 days after infection so that these patients rapidly developed acute respiratory distress syndrome, respiratory failure, multiple organ failure and even death [65].

More than half of the patients with dyspnea are bound to develop to severe cases requiring intensive care [65, 67, 69]. However, many critical patients failed early attempts at weaning from invasive mechanical ventilation so that the time of ICU stay appeared to be very long [65, 79]. This is surprising since most of them have recovered from pneumonia.

Several researchers also noticed that more than 50% of ICU patients exhibited dissociation between the mechanical characteristics of the respiratory system and the severity of hypoxemia [80–81]. In these patients, the compliance of the respiratory system and the amount of gas in the lung were both in the normal range. This is strange and has rarely been reported in other forms of acute respiratory distress syndrome [80–81, 82].

Respiratory viral infection can cause inflammatory changes and stimulate the sensory receptors located in the respiratory system, and hypoxemia can stimulate the glomus cells in the carotid and aortic bodies. The resultant impulses in these sensory structures are transmitted to and processed through the respiratory center located in the brainstem. The accommodative demands from the brainstem are then transmitted down to the phrenic nerves and diaphragm and cause increased ventilation. Meanwhile, the enhanced activity of respiratory center is transmitted up to the cerebral cortex, producing a subjective feeling of shortness of breath [83–84]. As an important warning signal of self-awareness, the incidence of dyspnea is significantly lower in COVID-19 patients than that in patients infected with many other respiratory viruses, such as Middle East Respiratory Syndrome (MERS)-CoV (69%), respiratory syncytial virus (95%), and influenza virus (82%) [85–86].

Autopsy studies show that the lungs of COVID-19 patients are characterized by diffuse alveolar damage with hyaline membrane formation, pneumocyte activation, microvascular thrombi, lymphocytic inflammation, and proteinaceous edema [87–88]. The exudation and fibrosis in terminal bronchioles and alveolar walls may lead to poor diffusion of oxygen across the alveolar barrier, while the increased thrombogenesis in pulmonary microvessels may aggravate hypoxemia. However, no evidence shows that these changes can cause blunting of dyspnea [84]. According to a case series study reported by Guan et al., among 1099 COVID-19 patients requiring hospital care or ICU admission, 23% and 12% had normal chest radiographic observations, respectively [74]. Moreover, some patients with acute respiratory distress did not show any evidence of pulmonary thromboembolism [23]. These data indicate that the acute respiratory failure induced by SARS-CoV-2 infection cannot be explained only by the pulmonary changes [89].

Respiratory failure may be caused by disturbance of any part of the respiratory movement, including the respiratory center, nerves, muscles, thorax, airways, and lungs. As discussed in detail below, increasing evidence shows that either or both PNS and CNS are involved in the respiratory failure of COVID-19 patients.

Neuromuscular dysfunction is associated with respiratory manifestations in some COVID-19 patients

Involvement of the PNS after SARS-CoV-2 infection includes anosmia, dysgeusia, Guillain-Barré Syndrome (GBS), myasthenia gravis myositis, myalgia, rhabdomyolysis, muscle wasting, and critical-ill myopathy [4, 43]. Rifino et al. performed a retrospective study on 137 COVID-19 patients with neurologic manifestations, and found that patients with PNS involvement more frequently developed severe

acute respiratory distress syndrome compared to patients with altered mental status or cerebrovascular disease [26]. There exists strong evidence supporting that respiratory nerves and/or muscles are involved in the acute respiratory failure of COVID-19 patients.

The diaphragm is the main inspiratory muscle, whose abnormalities affect coughing and expectoration, and result in a significant decrease in respiratory volume [90]. Phrenic nerves originate from spinal motoneurons at the levels of C3 ~ C5, which are regulated by spinal descending pathways crossing the levels of C1 ~ C2. Damage to the neural circuit controlling the diaphragm causes rapid deterioration of respiratory mechanics [91]. In support of this, Maurier et al. reported a 58-year-old female with COVID-19 who showed fever, dysgeusia and anosmia at the onset and rapidly developed progressive dyspnea due to phrenic paralysis [92]. Of note, this patient did not show any cardiac, pleural, parenchymal or pulmonary abnormalities, and creatinine phosphate kinase levels were also normal. Borroni *et al*. reported two COVID-19 patients with focal diaphragmatic myoclonus [93]. Electroencephalogram (EEG) showed no structural damage in the CNS in case 1, but revealed lateralized periodic discharges in the brain in case 2. Interestingly, the periodic discharges were closely correlated with the diaphragmatic myoclonic movements in this patient.

Diaphragmatic weakness has been widely described in COVID-19 patients with GBS, among whom quite a few developed severe respiratory failure [94]. Rajdev et al. reported a 36-year-old man who was diagnosed with COVID-19-associated GBS [95]. Although chest imaging showed that the lung lesions were recovering, he developed acute respiratory failure due to neuromuscular weakness caused by bulbar palsy. Patients with GBS usually have concomitant diaphragmatic weakness, which leads to atelectasis in the base of the lung, resulting in decreased lung compliance and increased intrapulmonary shunt. These changes, together with pulmonary infection, might induce a severe decline in lung volume and rapid deterioration of hypoxemia in COVID-19 patients [94].

The diaphragm was also frequently affected in ICU patients due to critical illness and mechanical ventilation [96–97]. Although required for many patients with acute respiratory failure, invasive mechanical ventilation can partially or completely unload respiratory muscles and silence the respiratory centers in the brainstem, leading to the inactivity of the diaphragm [98–99].

To date, a large amount of clinical data show that SARS-CoV-2 infection is often associated with acute neuromuscular dysfunction [5, 38]. Furthermore, neuromuscular dysfunction has been reported to be an important cause of acute respiratory distress syndrome in COVID-19 patients with minimal chest imaging findings [100].

Damage to the respiratory-related neural loops is associated with respiratory manifestations in some COVID-19 patients

Anosmia and dysgeusia are the most common PNS symptoms [43], indicating that SARS-CoV-2 infection may reduce the sensitivity of chemosensory reflexes [101–102]. Carotid/aortic bodies and bronchopulmonary C-fibers play a pivotal role in monitoring CO_2, H^+ and O_2^+ in the blood, and therefore damage to these structures has been suggested to be responsible for the absence of the sensation of dyspnea [103].

Carotid and aortic bodies are specialized sensory structures in arteries, where the cellular receptor for SARS-CoV-2, ACE2, is also present [104]. SARS-CoV-2 may directly invade the glomus cells in the carotid and aortic bodies or indirectly damage their sensory function due to the systemic inflammatory response and/or hypercoagulable condition in the blood. However, less than 1% of COVID-19 patients exhibited a detectable level of SARS-CoV-2 in the blood [105, 106], and the infection of carotid/aortic bodies has not yet been confirmed [58].

The affection of bronchopulmonary C-fibers has previously been reported to contribute to the respiratory failure induced by other respiratory viruses by abrogating the sensory transmission from lungs and respiratory airways [103, 107–108]. However, it is unclear whether this happens during SARS-CoV-2 infection.

Mechano- and chemoreceptors play a monitoring role in the lung and lower respiratory airways, while the respiratory reflex is triggered and controlled primarily by the respiratory center located in the brainstem. The brainstem is comprised of many important structures, which are essential for breathing, heart rate, blood pressure control, digestion, etc. These anatomical connections make the brainstem an easily accessible CNS target for SARS-CoV-2 from peripheral infection sites [1, 109]. In support of this, Lukiw et al. reported that the expression level of ACE2 was the highest in brainstem among 21 different brain regions in humans [110].

The brainstem has been reported to be highly infected with SARS-CoV [111] and MERS-COV [112]. In animal experiments, SARS-CoV and human CoV OC43

have been shown to enter the olfactory bulb after exposure to the nasal route and subsequently invade the CNS, including the brainstem [111, 113]. Considering the high similarity between SARS-CoV and SARS-CoV-2, we previously proposed that the potential infection of the brainstem may play a role in the acute respiratory failure of patients with COVID-19 [1–2].

Clinical evidence for involvement of the brainstem in COVID-19

In line with possible involvement of the brainstem, a case series study on COVID-19 patients younger than 18 years with neurological symptoms reported brainstem signs such as dysarthria or dysphagia in 2 cases (2/18) [114]. Similar findings were reported in some old patients [32, 35, 37]. However, absent or impaired brainstem reflexes are more common in severe patients who have been diagnosed with COVID-19-associated encephalomyelitis or encephalopathy [28, 50, 115].

Involvement of the brainstem was supported with neuroimaging findings in some COVID-19 patients. Wong et al. reported a 40-year-old man in England, who developed acute brainstem dysfunction after SARS-CoV-2 infection [116]. MRI scans revealed inflammatory changes in the brainstem in this case. Virhammar et al. described a 55-year-old female with acute necrotizing encephalopathy [28]. The CSF sample from this patient was positive for SARS-CoV-2. MRI scans revealed abnormal changes in several brain regions, including the brainstem. To date, abnormal imaging changes of the brainstem have been widely documented in COVID-19 patients with GBS [117], necrotizing encephalopathy [10, 28], and encephalomyelitis [118–121].

As stated earlier, it is difficult for many ICU patients with COVID-19 to withdraw invasive mechanical ventilation, even if their pulmonary infections have recovered [65, 80–81]. Related to this, Koutroumanidis et al. found that 5 of 13 ICU patients with COVID-19-associated encephalopathy had alpha coma EEG pattern [122]. Alpha coma is typically associated with the lesions located in the brainstem reticular formation [123]. Therefore, the relatively high incidence of alpha coma in severe patients with COVID-19 indicates that brainstem injury may be an important reason why they were difficult to get rid of invasive mechanical ventilation [122].

As a possible mechanism, the nerve endings within the olfactory neuroepithelium have been considered an entry point for SARS-CoV-2 to infect the brainstem [106]. Consistently, the mechanism underlying COVID-19-related olfactory dysfunction was reported to be obviously different from patients in acute colds, and may reflect, at least to some extent, a specific involvement at the level of CNS [124].

Eliezer et al. reported a female with COVID-19 who presented an acute loss of olfactory function without nasal obstruction. In this patient, CT and MRI analysis showed bilateral inflammatory obstruction in the olfactory clefts [125]. In a postmortem brain MRI study, Coolen et al. reported asymmetric olfactory bulbs in 4 of 19 patients with COVID-19 [126].

In a retrospective cohort study, Lin et al. reported that among 51 COVID-19 patients with MRI examinations 26 (51%) displayed acute or subacute findings in the CNS, including cranial nerve abnormalities (6) and critical illness-associated microbleeds (3). Of note, four patients displayed abnormally increased olfactory bulb signals suggesting olfactory neuritis, which might be related to the anosmia experienced by these patients [127].

In a prospective study, Lu et al. used MRI to evaluate the brains of 60 patients who had recovered from SARS-CoV-2 infection, and found that the volume of olfactory cortices was significantly increased in these patients [52]. Of note, 41 patients (68.33%) showed neurological symptoms during SARS-CoV-2 infection, and 30 (50%) still had neurological symptoms even though they had recovered 3 months after infection.

Autopsy evidence for involvement of the brainstem in COVID-19

Convincing evidence for involvement of the brainstem after SARS-CoV-2 infection has recently been reported in postmortem studies [44]. Among the published autopsy studies, neuropathological alterations were observed in the brainstem in 78 of 134 examined patients, including 18 with vascular accidents in the brainstem [54–55, 58, 128, 129–130], 15 with hypoxic injury in the brainstem [37, 54, 56, 63], and 65 with microgliosis/lymphocytic infiltration in the brainstem [36–37, 56–57, 62–63]. Among these cases, some had two or more types of these neuropathological changes. SARS-CoV-2 RNA and viral proteins were detected in the brainstem in 16/49 (32.7%) and 18/71 (25.3%) cases, respectively. The positive detection of SARS-CoV-2 was much higher in patients who showed microgliosis and/or lymphocytic infiltration in the brainstem, relative to patients with vascular accidents or hypoxemic lesions in the brainstem [32, 37, 54, 56, 60–61, 131].

The first autopsy study on the brainstem was published online on May 13, 2020 by Bulfamante et al. [131]. In

this study, a 54-year-old man who died from COVID-19 was observed to have severe degeneration in the neural tissues along the pathway from the olfactory nerve to the brainstem. Thereafter, von Weyhern et al. performed a more detailed postmortem study on the brainstem in 6 COVID-19 patients in Apr, 2020 [63], and they found that neuronal degeneration was extensively present in the brainstem in all 4 examined cases. Although no detection of SARS-CoV-2 was performed in the neural tissues in the two studies, the predominant involvement of the brainstem could not be attributed to only hypoxemia or hemorrhages.

Direct invasion of SARS-CoV-2 into the brainstem was reported in three autopsy studies in Jun, 2020 [32, 60–61]. Meinhardt et al. performed an autopsy study on 32 COVID-19 patients in Berlin, Germany, and detected SARS-CoV-2 RNA in the brainstem in 4 of 23 cases [32]. Menter et al. performed an autopsy study on 21 COVID-19 patients in Switzerland, and found SARS-CoV-2 RNA in the brainstem in all 4 cases examined [60]. Solomon et al. performed an autopsy study on 18 patients in Boston, USA, and found SARS-RNA in the brainstem in 3 of 18 cases [61].

On Oct 5, Matschke et al. published an autopsy study on the brainstem in 43 COVID-19 patients in *Lancet Neurology* [37]. Among the patients, 37 (86%) showed abnormal changes, including astrogliosis and/or microgliosis, in all assessed brain regions, but microglial activation and lymphocytic infiltration were the most severe in the brainstem and cerebellum. SARS-CoV-2 RNA was detected in the brains of 21 (53%) of 40 tested patients including the brainstem from 4 patients. Immunohistochemical staining revealed that the nucleocapsid protein of SARS-CoV-2 was present in neuron-like cells in the medulla oblongata and in the cranial nerves which originated from the lower brainstem.

In several studies, the presence of SARS-CoV-2 in the CNS was confirmed with different detection techniques [31–32, 37]. However, in some cases, positive results obtained by PCR tests could not be corroborated with *in situ* hybridization or immunohistochemistry using the same samples [54, 61]. Interestingly, in quite a few patients with negative PCR tests, SARS-CoV-2 was detectable in the same brain areas with immunohistochemistry [37].

The brainstem infection with SARS-CoV-2 is also supported with animal experiments. Deer mice, the most studied and abundant mammals in North America, are susceptible to SARS-CoV-2 infection because their ACE2 receptor shares 17 of the 20 critical residues for SARS-CoV-2 binding. As reported in COVID-19 patients, intranasal inoculation with SARS-CoV-2 caused respiratory, digestive and neurological infections in deer mice [132]. In the CNS of infected deer mice, SARS-CoV-2 antigen has been detected in a variety of brain areas, including the olfactory bulb and brainstem.

Therefore, both autopsy and animal studies indicate that the brainstem is one of the primary CNS targets of SARS-CoV-2, which may be the dominant reason for the unusually rapidly deteriorative respiratory function in some COVID-19 patients. The evidence currently available shows that SARS-CoV-2 can invade the brainstem in a retrograde manner via multiple nerve routes, including the olfactory, trigeminal, glossopharyngeal, and vagus nerves [37, 132].

The significance of acknowledging the neuroinvasive potential of SARS-CoV-2

Ever since their discovery in the late 1960s, the ability of CoVs to infect humans had been neglected by the international medical community [133]. Although two unexpected COVID pandemics, triggered by SARS-CoV and MERS-CoV, respectively, recall people's interest in CoV-infections, the neuroinvasive propensity of CoVs has still not attracted enough attention over the last 20 years. Unlike SARS-CoV and MERS-CoV, the rapid global spread of SARS-CoV-2 has posed an urgent and serious threat to public health.

As the counterpart of SARS-CoV-2, the pathogenesis of SARS-CoV infection remains poorly understood [134]. Similarly, a comprehensive understanding of SARS-CoV-2 is also lacking. Therefore, understanding the neuroinvasive potential of SARS-CoV-2 is of great significance for the prevention, treatment and prognosis of SARS-CoV-2 infection.

During the outbreak of COVID-19, the mortality of ICU patients with neurological problems was reported to be higher than that of patients without neurological symptoms [135]. Similarly, experimental studies show that the neuroinvasion of SARS-CoV-2 dramatically increased the mortality of infected animals [136]. Given this, SARS-CoV-2 infection in human beings should not be allowed to develop without treatment. As an example of the opposite, it has been reported that two-thirds of ICU patients in some hospitals were directly admitted from home [137].

Vaccination is considered the best option. However, before effective vaccines are available, wearing masks is undoubtedly a simple and effective measure against SARS-CoV-2 transmission [138], since it protects against invasion of the virus into the CNS from the respiratory tract and lung.

It is known that the virus in neurons can escape from the surveillance of the immune system, especially at the early stage of infection. The initially infected neurons eventually become apoptotic or are cleared by immune cells, prior to which virus progeny may have spread to other healthy neurons. The trans-neuronal transmission of CoVs makes it difficult to completely eliminate the virus from the CNS [139]. Therefore, the CNS infection of SARS-CoV-2 should be given antiviral treatment as soon as possible.

Chloroquine and hydroxychloroquine, which have been clinically used in COVID-19 patients, were reported to exhibit limited CNS penetration [140]. These drugs interfere with the glycosylation of ACE2 and therefore disturb the interaction of ACE2 with the spike protein of SARS-CoV-2. In addition, they prevent the endocytosis and subsequent vesicular trafficking of SARS-CoV-2 by endosomal alkalization [141].

During the epidemic of COVID-19, some researchers noticed that the patients who had been treated with adamantanes did not develop clinical diseases [142]. In these patients, adamantanes were initially used to treat the underlying neurologic disorders such as multiple sclerosis and Parkinson's disease. Adamantanes are known to possess an antiviral capability by binding a pore formed by SARS-CoV protein E and by interfering with the lysosomal phase of SARS-CoV infection. Of note, they can penetrate the blood-brain barrier, therefore may be considered a candidate to protest against the replication of SARS-CoV-2 in the CNS.

Previous studies reported that inhibition of tubulin polymerization hindered the retrograde axonal transport of poliomyelitis virus along infected peripheral nerves [143]. Therefore, some microtubule-associated inhibitors that have the capacity of penetrating the blood-brain barrier may be considered candidates to inhibit SARS-CoV-2 infection in the nervous system [106].

After replication in the CNS, progeny virions were exocytosed from host neuronal cells, and entered the next-order neurons by endocytosis [144]. One of the treatment alternatives available for COVID-19 is administration of anti-SARS-CoV-2 antibodies in plasma. Of interest, previous studies on West Nile virus showed that neutralizing antibodies could prevent viruses from spreading from neuron to neuron [145–146].

To date, there are some candidate drugs that can be tested to stop the CNS infection of SARS-CoV-2 [140, 147]. According to action sites, the drugs against neurotropic viruses can be divided into at least four kinds: blocking the invasion, transportation, replication, and release of viruses, respectively. With respect to the neurotropism of SARS-CoV-2, the CNS penetration ability of drugs is a critical factor for the treatment of brain infection. However, it should be noted that a kind of antiviral drugs alone may not be enough to stop the infection. For example, inhibiting virus transportation cannot alter the redistribution and replication of viruses. Therefore, it is recommended to combine two or more antiviral drugs to interfere with different stages of the life circle of viruses in the CNS.

Experimental studies on HCoV-OC43 show that the presence of CoV RNAs might last for at least one year without being acutely toxic in the brains of infected mice that had survived [148]. This suggests that CoVs can establish a persistent infection within the CNS of their hosts, which significantly increases the risk of long-term disability [149]. Consistent with this hypothesis, neuropsychiatric or neurocognitive disorders have been reported in some patients who had recovered from acute SARS-CoV-2 infection [150]. It is noteworthy that the young Japanese man, who was confirmed as the first case of meningitis/encephalitis [24] and has recovered from COVID-19, was found to develop retrograde amnesia and cannot recall what happened to him during his own infection [151].

Since the impact of SARS-CoV-2 infection on the nervous system may last for a long time, it is necessary to follow up the neurological changes of discharged patients and develop appropriate neurorehabilitation measures.

AUTHOR CONTRIBUTIONS

Y.C. Li conceived and designed the study. B.H. Tan, J.M. Liu, and Y.C. Li searched the literature, interpreted the data and wrote the manuscript. Y. Gui made the figures. S. Wu and J.L. Suo helped in the discussion and editing during writing of the manuscript. All authors critically reviewed and approved the final version of the paper.

CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

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REFERENCES

1. Li YC, Bai WZ, Hashikawa T. The neuroinvasive potential of SARS-CoV2 may play a role in the

respiratory failure of COVID-19 patients. J Med Virol. 2020; 92:552–555.
https://doi.org/10.1002/jmv.25728
PMID:32104915

2. Li YC, Bai WZ, Hashikawa T. Response to Commentary on "The neuroinvasive potential of SARS-CoV-2 may play a role in the respiratory failure of COVID-19 patients". J Med Virol. 2020; 92:707–709.
https://doi.org/10.1002/jmv.25824
PMID:32246783

3. Chigr F, Merzouki M, Najimi M. Comment on "The neuroinvasive potential of SARS-CoV-2 may p0lay a role in the respiratory failure of COVID-19 patients". J Med Virol. 2020; 92:703–704.
https://doi.org/10.1002/jmv.25960
PMID:32352575

4. Román GC, Spencer PS, Reis J, Buguet A, Faris MEA, Katrak SM, Láinez M, Medina MT, Meshram C, Mizusawa H, Öztürk S, Wasay M, and WFN Environmental Neurology Specialty Group. The neurology of COVID-19 revisited: A proposal from the Environmental Neurology Specialty Group of the World Federation of Neurology to implement international neurological registries. J Neurol Sci. 2020; 414:116884.
https://doi.org/10.1016/j.jns.2020.116884
PMID:32464367

5. Mao L, Jin H, Wang M, Hu Y, Chen S, He Q, Chang J, Hong C, Zhou Y, Wang D, Miao X, Li Y, Hu B. Neurologic Manifestations of Hospitalized Patients With Coronavirus Disease 2019 in Wuhan, China. JAMA Neurol. 2020; 77:683–690.
https://doi.org/10.1001/jamaneurol.2020.1127
PMID:32275288

6. Romero-Sánchez CM, Díaz-Maroto I, Fernández-Díaz E, Sánchez-Larsen Á, Layos-Romero A, García-García J, González E, Redondo-Peñas I, Perona-Moratalla AB, Del Valle-Pérez JA, Gracia-Gil J, Rojas-Bartolomé L, Feria-Vilar I, et al. Neurologic manifestations in hospitalized patients with COVID-19: The ALBACOVID registry. Neurology. 2020; 95:e1060–e1070.
https://doi.org/10.1212/wnl.0000000000009937
PMID:32482845

7. Pinna P, Grewal P, Hall JP, Tavarez T, Dafer RM, Garg R, Osteraas ND, Pellack DR, Asthana A, Fegan K, Patel V, Conners JJ, John S, et al. Neurological manifestations and COVID-19: Experiences from a tertiary care center at the Frontline. J Neurol Sci. 2020; 415:116969.
https://doi.org/10.1016/j.jns.2020.116969
PMID:32570113

8. Karadaş Ö, Öztürk B, Sonkaya AR. A prospective clinical study of detailed neurological manifestations in patients with COVID-19. Neurol Sci. 2020; 41:1991–1995.
https://doi.org/10.1007/s10072-020-04547-7
PMID:32588367

9. Cebrián J, Gonzalez-Martinez A, García-Blanco MJ, Celdrán-Vivancos D, Palacios EL, Reig-Roselló G, Casado-Fernández L, Vivancos J, Gago-Veiga AB. Headache and impaired consciousness level associated with SARS-CoV-2 in CSF: A case report. Neurology. 2020; 95:266–268.
https://doi.org/10.1212/wnl.0000000000010213
PMID:32641532

10. Delamarre L, Gollion C, Grouteau G, Rousset D, Jimena G, Roustan J, Gaussiat F, Aldigé E, Gaffard C, Duplantier J, Martin C, Fourcade O, Bost C, et al. COVID-19-associated acute necrotising encephalopathy successfully treated with steroids and polyvalent immunoglobulin with unusual IgG targeting the cerebral fibre network. J Neurol Neurosurg Psychiatry. 2020; 91:1004–1006.
https://doi.org/10.1136/jnnp-2020-323678
PMID:32651243

11. Destras G, Bal A, Escuret V, Morfin F, Lina B, Josset L, and COVID-Diagnosis HCL Study Group. Systematic SARS-CoV-2 screening in cerebrospinal fluid during the COVID-19 pandemic. Lancet Microbe. 2020; 1:e149.
https://doi.org/10.1016/s2666-5247(20)30066-5
PMID:32835345

12. Domingues RB, Mendes-Correa MC, de Moura Leite FBV, Sabino EC, Salarini DZ, Claro I, Santos DW, de Jesus JG, Ferreira NE, Romano CM, Soares CAS. First case of SARS-COV-2 sequencing in cerebrospinal fluid of a patient with suspected demyelinating disease. J Neurol. 2020; 267:3154–3156.
https://doi.org/10.1007/s00415-020-09996-w
PMID:32564153

13. Duong L, Xu P, Liu A. Meningoencephalitis without respiratory failure in a young female patient with COVID-19 infection in Downtown Los Angeles, early April 2020. Brain Behav Immun. 2020; 87:33.
https://doi.org/10.1016/j.bbi.2020.04.024
PMID:32305574

14. Edén A, Kanberg N, Gostner J, Fuchs D, Hagberg L, Andersson LM, Lindh M, Price RW, Zetterberg H, Gisslén M. CSF biomarkers in patients with COVID-19 and neurological symptoms: A case series. Neurology. 2021; 96:e294–e300.
https://doi.org/10.1212/wnl.0000000000010977
PMID:33004602

15. Fadakar N, Ghaemmaghami S, Masoompour SM, Shirazi Yeganeh B, Akbari A, Hooshmandi S, Ostovan VR. A First Case of Acute Cerebellitis Associated with Coronavirus Disease (COVID-19): a Case Report and Literature Review. Cerebellum. 2020; 19:911–914.

https://doi.org/10.1007/s12311-020-01177-9
PMID:32737799

16. Färber K, Stäbler P, Getzinger T, Uhlig T. [Suspected sepsis in a 10-week-old infant and SARS-CoV-2 detection in cerebrospinal fluid and pharynx]. [Article in German]. Monatsschr Kinderheilkd. 2020. [Epub ahead of print]. https://doi.org/10.1007/s00112-020-00942-8 PMID:32836394

17. Gigli GL, Vogrig A, Nilo A, Fabris M, Biasotto A, Curcio F, Miotti V, Tascini C, Valente M. HLA and immunological features of SARS-CoV-2-induced Guillain-Barré syndrome. Neurol Sci. 2020; 41:3391–3394. https://doi.org/10.1007/s10072-020-04787-7 PMID:33006723

18. Helms J, Kremer S, Merdji H, Schenck M, Severac F, Clere-Jehl R, Studer A, Radosavljevic M, Kummerlen C, Monnier A, Boulay C, Fafi-Kremer S, Castelain V, et al. Delirium and encephalopathy in severe COVID-19: a cohort analysis of ICU patients. Crit Care. 2020; 24:491. https://doi.org/10.1186/s13054-020-03200-1 PMID: 32771053

19. Huang YH, Jiang D, Huang JT. SARS-CoV-2 Detected in Cerebrospinal Fluid by PCR in a Case of COVID-19 Encephalitis. Brain Behav Immun. 2020; 87:149. https://doi.org/10.1016/j.bbi.2020.05.012 PMID:32387508

20. Kamal YM, Abdelmajid Y, Al Madani AAR. Cerebrospinal fluid confirmed COVID-19-associated encephalitis treated successfully. BMJ Case Rep. 2020; 13:e237378. https://doi.org/10.1136/bcr-2020-237378 PMID:32938656

21. Khodamoradi Z, Hosseini SA, Gholampoor Saadi MH, Mehrabi Z, Sasani MR, Yaghoubi S. COVID-19 meningitis without pulmonary involvement with positive cerebrospinal fluid PCR. Eur J Neurol. 2020; 27:2668–2669. https://doi.org/10.1111/ene.14536 PMID:32926584

22. Kremer S, Lersy F, de Sèze J, Ferré JC, Maamar A, Carsin-Nicol B, Collange O, Bonneville F, Adam G, Martin-Blondel G, Rafiq M, Geeraerts T, Delamarre L, et al. Brain MRI Findings in Severe COVID-19: A Retrospective Observational Study. Radiology. 2020; 297:E242–E251. https://doi.org/10.1148/radiol.2020202222 PMID:32544034

23. Mardani M, Nadji SA, Sarhangipor KA, Sharifi-Razavi A, Baziboroun M. COVID-19 infection recurrence presenting with meningoencephalitis. New Microbes New Infect. 2020; 37:100732. https://doi.org/10.1016/j.nmni.2020.100732 PMID:32789020

24. Moriguchi T, Harii N, Goto J, Harada D, Sugawara H, Takamino J, Ueno M, Sakata H, Kondo K, Myose N, Nakao A, Takeda M, Haro H, et al. A first case of meningitis/encephalitis associated with SARS-Coronavirus-2. Int J Infect Dis. 2020; 94:55–58. https://doi.org/10.1016/j.ijid.2020.03.062 PMID:32251791

25. Novi G, Rossi T, Pedemonte E, Saitta L, Rolla C, Roccatagliata L, Inglese M, Farinini D. Acute disseminated encephalomyelitis after SARS-CoV-2 infection. Neurol Neuroimmunol Neuroinflamm. 2020; 7:e797. https://doi.org/10.1212/nxi.0000000000000797 PMID:32482781

26. Rifino N, Censori B, Agazzi E, Alimonti D, Bonito V, Camera G, Conti MZ, Foresti C, Frigeni B, Gerevini S, Grimoldi M, La Gioia S, Partziguian T, et al. Neurologic manifestations in 1760 COVID-19 patients admitted to Papa Giovanni XXIII Hospital, Bergamo, Italy. J Neurol. 2020; 7:1–8. https://doi.org/10.1007/s00415-020-10251-5 PMID:33026520

27. Saitta L, Molin A, Villani F, Insorsi A, Roccatagliata L, Inglese M, Bassetti M, Pelosi P, Castellan L, Gerevini S, Robba C, Patroniti N. Brain microvascular occlusive disorder in COVID-19: a case report. Neurol Sci. 2020; 41:3401–3404. https://doi.org/10.1007/s10072-020-04795-7 PMID:33034804

28. Virhammar J, Kumlien E, Fällmar D, Frithiof R, Jackmann S, Sköld MK, Kadir M, Frick J, Lindeberg J, Olivero-Reinius H, Ryttlefors M, Cunningham JL, Wikström J, et al. Acute necrotizing encephalopathy with SARS-CoV-2 RNA confirmed in cerebrospinal fluid. Neurology. 2020; 95:445–449. https://doi.org/10.1212/wnl.0000000000010250 PMID:32586897

29. Westhoff TH, Seibert FS, Bauer F, Stervbo U, Anft M, Doevelaar AAN, Rohn BJ, Winnekendonk G, Dittmer U, Schenker P, Vonbrunn E, Amann K, Viebahn R, et al. Allograft infiltration and meningoencephalitis by SARS-CoV-2 in a pancreas-kidney transplant recipient. Am J Transplant. 2020; 20:3216–3220. https://doi.org/10.1111/ajt.16223 PMID:32713123

30. Xiang P, Xu XM, Gao LL, Wang HZ, Xiong HF, Li RH. First Case of 2019 Novel Coronavirus Disease with Encephalitis. ChinaXiv. 2020; T202003.00015. http://www.xinhuanet.com/english/2020-03/05/c_138846783.htm

31. Paniz-Mondolfi A, Bryce C, Grimes Z, Gordon RE, Reidy J, Lednicky J, Sordillo EM, Fowkes M. Central nervous system involvement by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). J Med Virol. 2020; 92:699–702. https://doi.org/10.1002/jmv.25915 PMID:32314810

32. Meinhardt J, Radke J, Dittmayer C, Mothes R, Franz J, Laue M, Schneider J, Brünink S, Hassan O, Stenzel W, Windgassen M, Rößler L, Goebel HH, et al. Olfactory transmucosal SARS-CoV-2 invasion as port of Central Nervous System entry in COVID-19 patients. BioRxiv. 2020. https://doi.org/10.1101/2020.06.04.135012

33. Cantuti-Castelvetri L, Ojha R, Pedro LD, Djannatian M, Franz J, Kuivanen S, Kallio K, Kaya T, Anastasina M, Smura T, Levanov L, Szirovicza L, Tobi A, et al. Neuropilin-1 facilitates SARS-CoV-2 cell entry and provides a possible pathway into the central nervous system. bioRxiv. 2020. https://doi.org/10.1101/2020.06.07.137802

34. Puelles VG, Lütgehetmann M, Lindenmeyer MT, Sperhake JP, Wong MN, Allweiss L, Chilla S, Heinemann A, Wanner N, Liu S, Braun F, Lu S, Pfefferle S, et al. Multiorgan and Renal Tropism of SARS-CoV-2. N Engl J Med. 2020; 383:590–592. https://doi.org/10.1056/nejmc2011400 PMID:32402155

35. Hanley B, Naresh KN, Roufosse C, Nicholson AG, Weir J, Cooke GS, Thursz M, Manousou P, Corbett R, Goldin R, Al-Sarraj S, Abdolrasouli A, Swann OC, et al. Histopathological findings and viral tropism in UK patients with severe fatal COVID-19: a post-mortem study. Lancet Microbe. 2020; 1:e245–e253. https://doi.org/10.1016/s2666-5247(20)30115-4 PMID:32844161

36. Freij BJ, Gebara BM, Tariq R, Wang AM, Gibson J, El-Wiher N, Krasan G, Patek PM, Levasseur KA, Amin M, Fullmer JM. Fatal central nervous system co-infection with SARS-CoV-2 and tuberculosis in a healthy child. BMC Pediatr. 2020; 20:429. https://doi.org/10.1186/s12887-020-02308-1 PMID:32907595

37. Matschke J, Lütgehetmann M, Hagel C, Sperhake JP, Schröder AS, Edler C, Mushumba H, Fitzek A, Allweiss L, Dandri M, Dottermusch M, Heinemann A, Pfefferle S, et al. Neuropathology of patients with COVID-19 in Germany: a post-mortem case series. Lancet Neurol. 2020; 19:919–929. https://doi.org/10.1016/s1474-4422(20)30308-2 PMID:33031735

38. Khalili N, Haseli S, Bahrami-Motlagh H, Keshavarz E, Khalili N, Langroudi TF, Khameneh A, Taheri MS. Neurologic Involvement in COVID-19: Radiologists' Perspective. Acad Radiol. 2020; 27:1051–1053. https://doi.org/10.1016/j.acra.2020.04.035 PMID:32402785

39. Agarwal P, Ray S, Madan A, Tyson B. Neurological manifestations in 404 COVID-19 patients in Washington State. J Neurol. 2020. [Epub ahead of print]. https://doi.org/10.1007/s00415-020-10087-z PMID:32761507

40. Chacón-Aguilar R, Osorio-Cámara JM, Sanjurjo-Jimenez I, González-González C, López-Carnero J, Pérez-Moneo B. COVID-19: Fever syndrome and neurological symptoms in a neonate. An Pediatr (Engl Ed). 2020; 92:373–374. https://doi.org/10.1016/j.anpede.2020.04.001 PMID:32341945

41. Gutiérrez-Ortiz C, Méndez-Guerrero A, Rodrigo-Rey S, San Pedro-Murillo E, Bermejo-Guerrero L, Gordo-Mañas R, de Aragón-Gómez F, Benito-León J. Miller Fisher syndrome and polyneuritis cranialis in COVID-19. Neurology. 2020; 95:e601–e605. https://doi.org/10.1212/wnl.0000000000009619 PMID:32303650

42. Wang HY, Li XL, Yan ZR, Sun XP, Han J, Zhang BW. Potential neurological symptoms of COVID-19. Ther Adv Neurol Disord. 2020; 13:1756286420917830. https://doi.org/10.1177/1756286420917830 PMID:32284735

43. Pezzini A, Padovani A. Lifting the mask on neurological manifestations of COVID-19. Nat Rev Neurol. 2020; 16:636–644. https://doi.org/10.1038/s41582-020-0398-3 PMID:32839585

44. Liu JM, Tan BH, Wu S, Gui Y, Suo JL, Li YC. Evidence of central nervous system infection and neuroinvasive routes, as well as neurological involvement, in the lethality of SARS-CoV-2 infection. J Med Virol. 2020. [Epub ahead of print]. https://doi.org/10.1002/jmv.26570 PMID:33002209

45. Espíndola OM, Siqueira M, Soares CN, Lima MAS, Leite ACC, Araujo AQC, Brandão CO, Silva MTT. Patients with COVID-19 and neurological manifestations show undetectable SARS-CoV-2 RNA levels in the cerebrospinal fluid. Int J Infect Dis. 2020; 96:567–569. https://doi.org/10.1016/j.ijid.2020.05.123 PMID:32505878

46. Djukic M, Schmidt-Samoa C, Lange P, Spreer A, Neubieser K, Eiffert H, Nau R, Schmidt H. Cerebrospinal fluid findings in adults with acute Lyme neuroborreliosis. J Neurol. 2012; 259:630–636. https://doi.org/10.1007/s00415-011-6221-8 PMID:21898139

47. Nagel MA, Forghani B, Mahalingam R, Wellish MC, Cohrs RJ, Russman AN, Katzan I, Lin R, Gardner CJ, Gilden DH. The value of detecting anti-VZV IgG antibody in CSF to diagnose VZV vasculopathy. Neurology. 2007; 68:1069–1073. https://doi.org/10.1212/01.wnl.0000258549.13334.16 PMID:17287447

48. Song E, Chow RD, Jiang R, Zamecnik CR, Loudermilk RP, Dai Y, Liu F, Geng B, Chiarella J, Israelow B, Casanovas-Massana A, Ko AI, Ring A, et al. Immunologically distinct responses occur in the CNS of COVID-19 patients. bioRxiv. 2020. https://doi.org/10.1101/2020.09.11.293464 PMID:32935102

49. Alexopoulos H, Magira E, Bitzogli K, Kafasi N, Vlachoyiannopoulos P, Tzioufas A, Kotanidou A, Dalakas MC. Anti-SARS-CoV-2 antibodies in the CSF, blood-brain barrier dysfunction, and neurological outcome: Studies in 8 stuporous and comatose patients. Neurol Neuroimmunol Neuroinflamm. 2020; 7:e893. https://doi.org/10.1212/nxi.0000000000000893 PMID:32978291

50. Benameur K, Agarwal A, Auld SC, Butters MP, Webster AS, Ozturk T, Howell JC, Bassit LC, Velasquez A, Schinazi RF, Mullins ME, Hu WT. Encephalopathy and Encephalitis Associated with Cerebrospinal Fluid Cytokine Alterations and Coronavirus Disease, Atlanta, Georgia, USA, 2020. Emerg Infect Dis. 2020; 26:2016–2021. https://doi.org/10.3201/eid2609.202122 PMID:32487282

51. Bellon M, Schweblin C, Lambeng N, Cherpillod P, Vazquez J, Lalive PH, Schibler M, Deffert C. Cerebrospinal fluid features in SARS-CoV-2 RT-PCR positive patients. Clin Infect Dis. 2020;ciaa1165. [Epub ahead of print]. https://doi.org/10.1093/cid/ciaa1165 PMID:32770235

52. Lu Y, Li X, Geng D, Mei N, Wu PY, Huang CC, Jia T, Zhao Y, Wang D, Xiao A, Yin B. Cerebral Micro-Structural Changes in COVID-19 Patients - An MRI-based 3-month Follow-up Study. EClinicalMedicine. 2020; 25:100484. https://doi.org/10.1016/j.eclinm.2020.100484 PMID:32838240

53. Wang M, Li T, Qiao F, Wang L, Li C, Gong Y. Coronavirus disease 2019 associated with aggressive neurological and mental abnormalities confirmed based on cerebrospinal fluid antibodies: A case report. Medicine (Baltimore). 2020; 99:e21428. https://doi.org/10.1097/md.0000000000021428 PMID:32898993

54. Al-Dalahmah O, Thakur KT, Nordvig AS, Prust ML, Roth W, Lignelli A, Uhlemann AC, Miller EH, Kunnath-Velayudhan S, Del Portillo A, Liu Y, Hargus G, Teich AF, et al. Neuronophagia and microglial nodules in a SARS-CoV-2 patient with cerebellar hemorrhage. Acta Neuropathol Commun. 2020; 8:147. https://doi.org/10.1186/s40478-020-01024-2 PMID:32847628

55. Bihlmaier K, Coras R, Willam C, Grampp S, Jabari S, Eichhorn P, Haller F, Kuramatsu J, Schwab S, Castellanos I, Birkholz T, Schüttler J, Altmeppen J, et al. Disseminated Multifocal Intracerebral Bleeding Events in Three Coronavirus Disease 2019 Patients on Extracorporeal Membrane Oxygenation As Rescue Therapy. Crit Care Explor. 2020; 2:e0218. https://doi.org/10.1097/cce.0000000000000218 PMID:32984837

56. Fabbri VP, Foschini MP, Lazzarotto T, Gabrielli L, Cenacchi G, Gallo C, Aspide R, Frascaroli G, Cortelli P, Riefolo M, Giannini C, D'Errico A. Brain ischemic injury in COVID-19-infected patients: a series of 10 post-mortem cases. Brain Pathol. 2021; 31:205–210. https://doi.org/10.1111/bpa.12901 PMID:33002281

57. Jensen MP, Le Quesne J, Officer-Jones L, Teodòsio A, Thaventhiran J, Ficken C, Goddard M, Smith C, Menon D, Allinson KSJ. Neuropathological findings in two patients with fatal COVID-19. Neuropathol Appl Neurobiol. 2021; 47:17–25. https://doi.org/10.1111/nan.12662 PMID:32895961

58. Kantonen J, Mahzabin S, Mäyränpää MI, Tynninen O, Paetau A, Andersson N, Sajantila A, Vapalahti O, Carpén O, Kekäläinen E, Kantele A, Myllykangas L. Neuropathologic features of four autopsied COVID-19 patients. Brain Pathol. 2020; 30:1012–1016. https://doi.org/10.1111/bpa.12889 PMID:32762083

59. Remmelink M, De Mendonça R, D'Haene N, De Clercq S, Verocq C, Lebrun L, Lavis P, Racu ML, Trépant AL, Maris C, Rorive S, Goffard JC, De Witte O, et al. Unspecific post-mortem findings despite multiorgan viral spread in COVID-19 patients. Crit Care. 2020; 24:495. https://doi.org/10.1186/s13054-020-03218-5 PMID:32787909

60. Menter T, Haslbauer JD, Nienhold R, Savic S, Hopfer H, Deigendesch N, Frank S, Turek D, Willi N, Pargger H, Bassetti S, Leuppi JD, Cathomas G, et al. Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings in lungs and other organs suggesting vascular dysfunction. Histopathology. 2020; 77:198–209. https://doi.org/10.1111/his.14134 PMID:32364264

61. Solomon IH, Normandin E, Bhattacharyya S, Mukerji SS, Keller K, Ali AS, Adams G, Hornick JL, Padera RF Jr, Sabeti P. Neuropathological Features of Covid-19. N Engl J Med. 2020; 383:989–992. https://doi.org/10.1056/nejmc2019373 PMID:32530583

62. Schurink B, Roos E, Radonic T, Barbe E, Bouman CSC, de Boer HH, de Bree GJ, Bulle EB, Aronica EM, Florquin S, Fronczek J, Heunks LMA, de Jong MD, et al. Viral presence and immunopathology in patients with lethal COVID-19: a prospective autopsy cohort study. Lancet Microbe. 2020; 1:e290–e299. https://doi.org/10.1016/s2666-5247(20)30144-0 PMID:33015653

63. von Weyhern CH, Kaufmann I, Neff F, Kremer M. Early evidence of pronounced brain involvement in fatal COVID-19 outcomes. Lancet. 2020; 395:e109. https://doi.org/10.1016/s0140-6736(20)31282-4 PMID:32505222

64. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Med. 2020; 46:846–848. https://doi.org/10.1007/s00134-020-05991-x PMID:32125452

65. Huang C. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/s0140-6736(20)30183-5 PMID:31986264

66. Ñamendys-Silva SA. Respiratory support for patients with COVID-19 infection. Lancet Respir Med. 2020; 8:e18. https://doi.org/10.1016/s2213-2600(20)30110-7 PMID:32145829

67. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–513. https://doi.org/10.1016/s0140-6736(20)30211-7 PMID:32007143

68. Zhou Y, Zheng Y, Yang Q, Hu L, Liao J, Li X. Cohort study of chest CT and clinical changes in 29 patients with coronavirus disease 2019 (COVID-19). Eur Radiol. 2020; 30:6213–6220. https://doi.org/10.1007/s00330-020-07007-0 PMID:32591890

69. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, et al. Clinical Characteristics of 138 Hospitalized Patients With 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. JAMA. 2020; 323:1061–1069. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

70. Liu W, Tao ZW, Wang L, Yuan ML, Liu K, Zhou L, Wei S, Deng Y, Liu J, Liu HG, Yang M, Hu Y. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. Chin Med J (Engl). 2020; 133:1032–1038. https://doi.org/10.1097/cm9.0000000000000775 PMID:32118640

71. Zhang G, Hu C, Luo L, Fang F, Chen Y, Li J, Peng Z, Pan H. Clinical features and short-term outcomes of 221 patients with COVID-19 in Wuhan, China. J Clin Virol. 2020; 127:104364. https://doi.org/10.1016/j.jcv.2020.104364 PMID:32311650

72. Zhang R, Ouyang H, Fu L, Wang S, Han J, Huang K, Jia M, Song Q, Fu Z. CT features of SARS-CoV-2 pneumonia according to clinical presentation: a retrospective analysis of 120 consecutive patients from Wuhan city. Eur Radiol. 2020; 30:4417–4426. https://doi.org/10.1007/s00330-020-06854-1 PMID:32279115

73. Deng Y, Liu W, Liu K, Fang YY, Shang J, Zhou L, Wang K, Leng F, Wei S, Chen L, Liu HG. Clinical characteristics of fatal and recovered cases of coronavirus disease 2019 in Wuhan, China: a retrospective study. Chin Med J (Engl). 2020; 133:1261–1267. https://doi.org/10.1097/cm9.0000000000000824 PMID:32209890

74. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, et al. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020; 382:1708–1720. https://doi.org/10.1056/nejmoa2002032 PMID:32109013

75. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Zhou X, Du C, Zhang Y, Song J, Wang S, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. JAMA Intern Med. 2020; 180:934–943.

https://doi.org/10.1001/jamainternmed.2020.0994
PMID:32167524

76. Chen Q, Zheng Z, Zhang C, Zhang X, Wu H, Wang J, Wang S, Zheng C. Clinical characteristics of 145 patients with corona virus disease 2019 (COVID-19) in Taizhou, Zhejiang, China. Infection. 2020; 48:543–551.
https://doi.org/10.1007/s15010-020-01432-5
PMID:32342479

77. Li K, Wu J, Wu F, Guo D, Chen L, Fang Z, Li C. The Clinical and Chest CT Features Associated With Severe and Critical COVID-19 Pneumonia. Invest Radiol. 2020; 55:327–331.
https://doi.org/10.1097/rli.0000000000000672
PMID:32118615

78. Couzin-Frankel J. The mystery of the pandemic's 'happy hypoxia'. Science. 2020; 368:455–456.
https://doi.org/10.1126/science.368.6490.455
PMID:32355007

79. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, Cereda D, Coluccello A, Foti G, Fumagalli R, Iotti G, Latronico N, Lorini L, et al. Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. JAMA. 2020; 323:1574–1581.
https://doi.org/10.1001/jama.2020.5394
PMID:32250385

80. Gattinoni L, Chiumello D, Caironi P, Busana M, Romitti F, Brazzi L, Camporota L. COVID-19 pneumonia: different respiratory treatments for different phenotypes? Intensive Care Med. 2020; 46:1099–1102.
https://doi.org/10.1007/s00134-020-06033-2
PMID:32291463

81. Gattinoni L, Coppola S, Cressoni M, Busana M, Rossi S, Chiumello D. COVID-19 Does Not Lead to a "Typical" Acute Respiratory Distress Syndrome. Am J Respir Crit Care Med. 2020; 201:1299–1300.
https://doi.org/10.1164/rccm.202003-0817LE
PMID:32228035

82. Salehi S, Abedi A, Balakrishnan S, Gholamrezanezhad A. Coronavirus Disease 2019 (COVID-19): A Systematic Review of Imaging Findings in 919 Patients. AJR Am J Roentgenol. 2020; 215:87–93.
https://doi.org/10.2214/ajr.20.23034
PMID:32174129

83. Guyenet PG, Bayliss DA. Neural Control of Breathing and CO2 Homeostasis. Neuron. 2015; 87:946–961.
https://doi.org/10.1016/j.neuron.2015.08.001
PMID:26335642

84. Tobin MJ, Laghi F, Jubran A. Why COVID-19 Silent Hypoxemia Is Baffling to Physicians. Am J Respir Crit Care Med. 2020; 202:356–360.
https://doi.org/10.1164/rccm.202006-2157cp
PMID:32539537

85. Bertran Recasens B, Martinez-Llorens JM, Rodriguez-Sevilla JJ, Rubio MA. Lack of dyspnea in patients with Covid-19: another neurological conundrum? Eur J Neurol. 2020; 27:e40.
https://doi.org/10.1111/ene.14265
PMID:32301553

86. Cavallazzi R, Ramirez JA. Influenza and Viral Pneumonia. Clin Chest Med. 2018; 39:703–721.
https://doi.org/10.1016/j.ccm.2018.07.005
PMID:30390743

87. Wichmann D, Sperhake JP, Lütgehetmann M, Steurer S, Edler C, Heinemann A, Heinrich F, Mushumba H, Kniep I, Schröder AS, Burdelski C, de Heer G, Nierhaus A, et al. Autopsy Findings and Venous Thromboembolism in Patients With COVID-19: A Prospective Cohort Study. Ann Intern Med. 2020; 173:268–277.
https://doi.org/10.7326/m20-2003
PMID:32374815

88. Ackermann M, Verleden SE, Kuehnel M, Haverich A, Welte T, Laenger F, Vanstapel A, Werlein C, Stark H, Tzankov A, Li WW, Li VW, Mentzer SJ, et al. Pulmonary Vascular Endothelialitis, Thrombosis, and Angiogenesis in Covid-19. N Engl J Med. 2020; 383:120–128.
https://doi.org/10.1056/nejmoa2015432
PMID:32437596

89. Ylikoski J, Markkanen M, Mäkitie A. Pathophysiology of the COVID-19 - entry to the CNS through the nose. Acta Otolaryngol. 2020; 140:886–889.
https://doi.org/10.1080/00016489.2020.1773533
PMID:32597276

90. Angelini C, Siciliano G. Neuromuscular diseases and Covid-19: Advices from scientific societies and early observations in Italy. Eur J Transl Myol. 2020; 30:9032.
https://doi.org/10.4081/ejtm.2019.9032
PMID:32782765

91. Baig AM. Computing the Effects of SARS-CoV-2 on Respiration Regulatory Mechanisms in COVID-19. ACS Chem Neurosci. 2020; 11:2416–2421.
https://doi.org/10.1021/acschemneuro.0c00349
PMID:32600045

92. Maurier F, Godbert B, Perrin J. Respiratory Distress in SARS-CoV-2 without Lung Damage: Phrenic Paralysis Should Be Considered in COVID-19 Infection. Eur J Case Rep Intern Med. 2020; 7:001728.
https://doi.org/10.12890/2020_001728
PMID:32523929

93. Borroni B, Gazzina S, Dono F, Mazzoleni V, Liberini P, Carrarini C, Russo M, Pontolillo M, Vecchiet J, Onofrj

M, Bonanni L. Diaphragmatic myoclonus due to SARS-CoV-2 infection. Neurol Sci. 2020; 41:3471–3474. https://doi.org/10.1007/s10072-020-04766-y PMID:33090303

94. Galassi G, Marchioni A. Facing acute neuromuscular diseases during COVID-19 pandemic: focus on Guillain-Barré syndrome. Acta Neurol Belg. 2020; 120:1067–1075. https://doi.org/10.1007/s13760-020-01421-3 PMID:32696312

95. Rajdev K, Victor N, Buckholtz ES, Hariharan P, Saeed MA, Hershberger DM, Bista S. A Case of Guillain-Barré Syndrome Associated With COVID-19. J Investig Med High Impact Case Rep. 2020; 8:2324709620961198. https://doi.org/10.1177/2324709620961198 PMID:32981333

96. Dres M, Goligher EC, Heunks LMA, Brochard LJ. Critical illness-associated diaphragm weakness. Intensive Care Med. 2017; 43:1441–1452. https://doi.org/10.1007/s00134-017-4928-4 PMID:28917004

97. Dres M, Jung B, Molinari N, Manna F, Dubé BP, Chanques G, Similowski T, Jaber S, Demoule A. Respective contribution of intensive care unit-acquired limb muscle and severe diaphragm weakness on weaning outcome and mortality: a post hoc analysis of two cohorts. Crit Care. 2019; 23:370. https://doi.org/10.1186/s13054-019-2650-z PMID:31752937

98. Brosnahan SB, Jonkman AH, Kugler MC, Munger JS, Kaufman DA. COVID-19 and Respiratory System Disorders: Current Knowledge, Future Clinical and Translational Research Questions. Arterioscler Thromb Vasc Biol. 2020; 40:2586–2597. https://doi.org/10.1161/atvbaha.120.314515 PMID:32960072

99. Guarracino F, Vetrugno L, Forfori F, Corradi F, Orso D, Bertini P, Ortalda A, Federici N, Copetti R, Bove T. Lung, Heart, Vascular, and Diaphragm Ultrasound Examination of COVID-19 Patients: A Comprehensive Approach. J Cardiothorac Vasc Anesth. 2020. [Epub ahead of print]. https://doi.org/10.1053/j.jvca.2020.06.013 PMID:32624431

100. Nouri-Vaskeh M, Sharifi A, Khalili N, Zand R, Sharifi A. Dyspneic and non-dyspneic (silent) hypoxemia in COVID-19: Possible neurological mechanism. Clin Neurol Neurosurg. 2020; 198:106217. https://doi.org/10.1016/j.clineuro.2020.106217 PMID:32947193

101. Beltrán-Corbellini Á, Chico-García JL, Martínez-Poles J, Rodríguez-Jorge F, Natera-Villalba E, Gómez-Corral J, Gómez-López A, Monreal E, Parra-Díaz P, Cortés-Cuevas JL, Galán JC, Fragola-Arnau C, Porta-Etessam J, et al. Acute-onset smell and taste disorders in the context of Covid-19: a pilot multicenter PCR-based case-control study. Eur J Neurol. 2020; 27:1738–1741. https://doi.org/10.1111/ene.14273 PMID:32320508

102. Giacomelli A, Pezzati L, Conti F, Bernacchia D, Siano M, Oreni L, Rusconi S, Gervasoni C, Ridolfo AL, Rizzardini G, Antinori S, Galli M. Self-reported Olfactory and Taste Disorders in Patients With Severe Acute Respiratory Coronavirus 2 Infection: A Cross-sectional Study. Clin Infect Dis. 2020; 71:889–890. https://doi.org/10.1093/cid/ciaa330 PMID:32215618

103. Tan BH, Zhang Y, Gui Y, Wu S, Li YC. The possible impairment of respiratory-related neural loops may be associated with the silent pneumonia induced by SARS-CoV-2. J Med Virol. 2020; 92:2269–2271. https://doi.org/10.1002/jmv.26158 PMID:32525575

104. Fung ML. Expressions of angiotensin and cytokine receptors in the paracrine signaling of the carotid body in hypoxia and sleep apnea. Respir Physiol Neurobiol. 2015; 209:6–12. https://doi.org/10.1016/j.resp.2014.09.014 PMID:25266394

105. Chen X, Zhao B, Qu Y, Chen Y, Xiong J, Feng Y, Men D, Huang Q, Liu Y, Yang B, Ding J, Li F. Detectable Serum Severe Acute Respiratory Syndrome Coronavirus 2 Viral Load (RNAemia) Is Closely Correlated With Drastically Elevated Interleukin 6 Level in Critically Ill Patients With Coronavirus Disease 2019. Clin Infect Dis. 2020; 71:1937–1942. https://doi.org/10.1093/cid/ciaa449 PMID:32301997

106. Fenrich M, Mrdenovic S, Balog M, Tomic S, Zjalic M, Roncevic A, Mandic D, Debeljak Z, Heffer M. SARS-CoV-2 Dissemination Through Peripheral Nerves Explains Multiple Organ Injury. Front Cell Neurosci. 2020; 14:229. https://doi.org/10.3389/fncel.2020.00229 PMID:32848621

107. Zhuang J, Gao P, Pollock Z, Harrod KS, Xu F. Depressed Hypoxic and Hypercapnic Ventilatory Responses at Early Stage of Lethal Avian Influenza A Virus Infection in Mice. PLoS One. 2016; 11:e0147522. https://doi.org/10.1371/journal.pone.0147522 PMID:26808681

108. Zhuang J, Zang N, Ye C, Xu F. Lethal avian influenza A (H5N1) virus replicates in pontomedullary chemosensitive neurons and depresses hypercapnic ventilator response in mice. Am J Physiol Lung Cell Mol Physiol. 2019; 316:L525–L536. https://doi.org/10.1152/ajplung.00324.2018 PMID:30628490

109. Briguglio M, Bona A, Porta M, Dell'Osso B, Pregliasco FE, Banfi G. Disentangling the Hypothesis of Host Dysosmia and SARS-CoV-2: The Bait Symptom That Hides Neglected Neurophysiological Routes. Front Physiol. 2020; 11:671. https://doi.org/10.3389/fphys.2020.00671 PMID:32581854

110. Lukiw WJ, Pogue A, Hill JM. SARS-CoV-2 Infectivity and Neurological Targets in the Brain. Cell Mol Neurobiol. 2020. [Epub ahead of print]. https://doi.org/10.1007/s10571-020-00947-7 PMID:32840758

111. Netland J, Meyerholz DK, Moore S, Cassell M, Perlman S. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. J Virol. 2008; 82:7264–75. https://doi.org/10.1128/jvi.00737-08 PMID:18495771

112. Li K, Wohlford-Lenane C, Perlman S, Zhao J, Jewell AK, Reznikov LR, Gibson-Corley KN, Meyerholz DK, McCray PB Jr. Middle East Respiratory Syndrome Coronavirus Causes Multiple Organ Damage and Lethal Disease in Mice Transgenic for Human Dipeptidyl Peptidase 4. J Infect Dis. 2016; 213:712–722. https://doi.org/10.1093/infdis/jiv499 PMID:26486634

113. Dubé M, Le Coupanec A, Wong AHM, Rini JM, Desforges M, Talbot PJ. Axonal Transport Enables Neuron-to-Neuron Propagation of Human Coronavirus OC43. J Virol. 2018; 92:e00404–18. https://doi.org/10.1128/jvi.00404-18 PMID:29925652

114. Abdel-Mannan O, Eyre M, Löbel U, Bamford A, Eltze C, Hameed B, Hemingway C, Hacohen Y. Neurologic and Radiographic Findings Associated With COVID-19 Infection in Children. JAMA Neurol. 2020; 77:1440–45. https://doi.org/10.1001/jamaneurol.2020.2687 PMID:32609336

115. Khoo A, McLoughlin B, Cheema S, Weil RS, Lambert C, Manji H, Zandi MS, Morrow JM. Postinfectious brainstem encephalitis associated with SARS-CoV-2. J Neurol Neurosurg Psychiatry. 2020; 91:1013–1014. https://doi.org/10.1136/jnnp-2020-323816 PMID:32636212

116. Wong PF, Craik S, Newman P, Makan A, Srinivasan K, Crawford E, Dev D, Moudgil H, Ahmad N. Lessons of the month 1: A case of rhombencephalitis as a rare complication of acute COVID-19 infection. Clin Med (Lond). 2020; 20:293–294. https://doi.org/10.7861/clinmed.2020-0182 PMID:32371417

117. Sancho-Saldaña A, Lambea-Gil Á, Liesa JLC, Caballo MRB, Garay MH, Celada DR, Serrano-Ponz M. Guillain-Barré syndrome associated with leptomeningeal enhancement following SARS-CoV-2 infection. Clin Med (Lond). 2020; 20:e93–e94. https://doi.org/10.7861/clinmed.2020-0213 PMID:32518103

118. Afshar H, Yassin Z, Kalantari S, Aloosh O, Lotfi T, Moghaddasi M, Sadeghipour A, Emamikhah M. Evolution and resolution of brain involvement associated with SARS- CoV2 infection: A close Clinical - Paraclinical follow up study of a case. Mult Scler Relat Disord. 2020; 43:102216. https://doi.org/10.1016/j.msard.2020.102216 PMID:32464585

119. McCuddy M, Kelkar P, Zhao Y, Wicklund D. Acute Demyelinating Encephalomyelitis (ADEM) in COVID-19 Infection: A Case Series. Neurol India. 2020; 68:1192–1195. https://doi.org/10.4103/0028-3886.299174 PMID:33109874

120. Zambreanu L, Lightbody S, Bhandari M, Hoskote C, Kandil H, Houlihan CF, Lunn MP. A case of limbic encephalitis associated with asymptomatic COVID-19 infection. J Neurol Neurosurg Psychiatry. 2020; 91:1229–1230. https://doi.org/10.1136/jnnp-2020-323839 PMID:32661082

121. Zoghi A, Ramezani M, Roozbeh M, Darazam IA, Sahraian MA. A case of possible atypical demyelinating event of the central nervous system following COVID-19. Mult Scler Relat Disord. 2020; 44:102324. https://doi.org/10.1016/j.msard.2020.102324 PMID:32615528

122. Koutroumanidis M, Gratwicke J, Sharma S, Whelan A, Tan SV, Glover G. Alpha coma EEG pattern in patients with severe COVID-19 related encephalopathy. Clin Neurophysiol. 2021; 132:218–225. https://doi.org/10.1016/j.clinph.2020.09.008 PMID:33060058

123. Obeso JA, Iragui MI, Marti-Masso JF, Maravi E, Teijeira JM, Carrera N, Teijeria J. Neurophysiological

assessment of alpha pattern coma. J Neurol Neurosurg Psychiatry. 1980; 43:63–7. https://doi.org/10.1136/jnnp.43.1.63 PMID:7354359

124. Huart C, Philpott C, Konstantinidis I, Altundag A, Whitcroft KL, Trecca EMC, Cassano M, Rombaux P, Hummel T. Comparison of COVID-19 and common cold chemosensory dysfunction. Rhinology. 2020; 58:623–625. https://doi.org/10.4193/rhin20.251 PMID:32812014

125. Eliezer M, Hautefort C, Hamel AL, Verillaud B, Herman P, Houdart E, Eloit C. Sudden and Complete Olfactory Loss Function as a Possible Symptom of COVID-19. JAMA Otolaryngol Head Neck Surg. 2020; 146:674–675. https://doi.org/10.1001/jamaoto.2020.0832 PMID:32267483

126. Coolen T, Lolli V, Sadeghi N, Rovai A, Trotta N, Taccone FS, Creteur J, Henrard S, Goffard JC, Dewitte O, Naeije G, Goldman S, De Tiège X. Early postmortem brain MRI findings in COVID-19 non-survivors. Neurology. 2020; 95:e2016–e2027. https://doi.org/10.1212/wnl.0000000000010116 PMID:32546654

127. Lin E, Lantos JE, Strauss SB, Phillips CD, Campion TR Jr, Navi BB, Parikh NS, Merkler AE, Mir S, Zhang C, Kamel H, Cusick M, Goyal P, et al. Brain Imaging of Patients with COVID-19: Findings at an Academic Institution during the Height of the Outbreak in New York City. AJNR Am J Neuroradiol. 2020; 41:2001–2008. https://doi.org/10.3174/ajnr.a6793 PMID:32819899

128. Bradley BT, Maioli H, Johnston R, Chaudhry I, Fink SL, Xu H, Najafian B, Deutsch G, Lacy JM, Williams T, Yarid N, Marshall DA. Histopathology and ultrastructural findings of fatal COVID-19 infections in Washington State: a case series. Lancet. 2020; 396:320–332. https://doi.org/10.1016/s0140-6736(20)31305-2 PMID:32682491

129. Conklin J, Frosch MP, Mukerji S, Rapalino O, Maher M, Schaefer PW, Lev MH, Gonzalez RG, Das S, Champion SN, Magdamo C, Sen P, Harrold GK, et al. Cerebral Microvascular Injury in Severe COVID-19. medRxiv. 2020. https://doi.org/10.1101/2020.07.21.20159376 PMID:32743599

130. Keller E, Brandi G, Winklhofer S, Imbach LL, Kirschenbaum D, Frontzek K, Steiger P, Dietler S, Haeberlin M, Willms J, Porta F, Waeckerlin A, Huber M, et al. Large and Small Cerebral Vessel Involvement in Severe COVID-19: Detailed Clinical Workup of a Case Series. Stroke. 2020; 51:3719–3722. https://doi.org/10.1161/strokeaha.120.031224 PMID:33054673

131. Bulfamante G, Chiumello D, Canevini MP, Priori A, Mazzanti M, Centanni S, Felisati G. First ultrastructural autoptic findings of SARS -Cov-2 in olfactory pathways and brainstem. Minerva Anestesiol. 2020; 86:678–679. https://doi.org/10.23736/s0375-9393.20.14772-2 PMID:32401000

132. Fagre A, Lewis J, Eckley M, Zhan S, Rocha SM, Sexton NR, Burke B, Geiss BJ, Peersen O, Kading R, Rovnak J, Ebel GD, Tjalkens RB, et al. SARS-CoV-2 infection, neuropathogenesis and transmission among deer mice: Implications for reverse zoonosis to New World rodents. bioRxiv. 2020. https://doi.org/10.1101/2020.08.07.241810 PMID:32793912

133. Desforges M, Le Coupanec A, Brison E, Meessen-Pinard M, Talbot PJ. Neuroinvasive and neurotropic human respiratory coronaviruses: potential neurovirulent agents in humans. Adv Exp Med Biol. 2014; 807:75–96. https://doi.org/10.1007/978-81-322-1777-0_6 PMID:24619619

134. Gu J, Gong E, Zhang B, Zheng J, Gao Z, Zhong Y, Zou W, Zhan J, Wang S, Xie Z, Zhuang H, Wu B, Zhong H, et al. Multiple organ infection and the pathogenesis of SARS. J Exp Med. 2005; 202:415–424. https://doi.org/10.1084/jem.20050828 PMID:16043521

135. Phua J, Weng L, Ling L, Egi M, Lim CM, Divatia JV, Shrestha BR, Arabi YM, Ng J, Gomersall CD, Nishimura M, Koh Y, Du B, et al. Intensive care management of coronavirus disease 2019 (COVID-19): challenges and recommendations. Lancet Respir Med. 2020; 8:506–517. https://doi.org/10.1016/s2213-2600(20)30161-2 PMID:32272080

136. Jiang RD, Liu MQ, Chen Y, Shan C, Zhou YW, Shen XR, Li Q, Zhang L, Zhu Y, Si HR, Wang Q, Min J, Wang X, et al. Pathogenesis of SARS-CoV-2 in Transgenic Mice Expressing Human Angiotensin-Converting Enzyme 2. Cell. 2020; 182:50–58.e8. https://doi.org/10.1016/j.cell.2020.05.027 PMID:32516571

137. Bhatraju PK, Ghassemieh BJ, Nichols M, Kim R, Jerome KR, Nalla AK, Greninger AL, Pipavath S, Wurfel MM, Evans L, Kritek PA, West TE, Luks A, et al. Covid-19 in Critically Ill Patients in the Seattle Region - Case Series. N Engl J Med. 2020; 382:2012–2022. https://doi.org/10.1056/nejmoa2004500 PMID:32227758

138. Tirupathi R, Bharathidasan K, Palabindala V, Salim SA, Al-Tawfiq JA. Comprehensive review of mask utility and challenges during the COVID-19 pandemic. Infez Med. 2020; 28:57–63.
PMID:32532940

139. Rothe C, Schunk M, Sothmann P, Bretzel G, Froeschl G, Wallrauch C, Zimmer T, Thiel V, Janke C, Guggemos W, Seilmaier M, Drosten C, Vollmar P, et al. Transmission of 2019-nCoV Infection from an Asymptomatic Contact in Germany. N Engl J Med. 2020; 382:970–971.
https://doi.org/10.1056/nejmc2001468
PMID:32003551

140. Ong WY, Go ML, Wang DY, Cheah IK, Halliwell B. Effects of Antimalarial Drugs on Neuroinflammation-Potential Use for Treatment of COVID-19-Related Neurologic Complications. Mol Neurobiol. 2021; 58:106–117.
https://doi.org/10.1007/s12035-020-02093-z
PMID:32897518

141. Liu J, Cao R, Xu M, Wang X, Zhang H, Hu H, Li Y, Hu Z, Zhong W, Wang M. Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection *in vitro*. Cell Discov. 2020; 6:16.
https://doi.org/10.1038/s41421-020-0156-0
PMID:32194981

142. Brenner SR. The potential of memantine and related adamantanes such as amantadine, to reduce the neurotoxic effects of COVID-19, including ARDS and to reduce viral replication through lysosomal effects. J Med Virol. 2020; 92:2341–2342.
https://doi.org/10.1002/jmv.26030
PMID:32436995

143. Ohka S, Matsuda N, Tohyama K, Oda T, Morikawa M, Kuge S, Nomoto A. Receptor (CD155)-dependent endocytosis of poliovirus and retrograde axonal transport of the endosome. J Virol. 2004; 78:7186–98.
https://doi.org/10.1128/jvi.78.13.7186-7198.2004
PMID:15194795

144. Li YC, Bai WZ, Hirano N, Hayashida T, Taniguchi T, Sugita Y, Tohyama K, Hashikawa T. Neurotropic virus tracing suggests a membranous-coating-mediated mechanism for transsynaptic communication. J Comp Neurol. 2013; 521:203–12.
https://doi.org/10.1002/cne.23171
PMID:22700307

145. Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, Gorlatov S, Mehlhop E, Marri A, Chung KM, Ebel GD, Kramer LD, Fremont DH, et al. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat Med. 2005; 11:522–30.
https://doi.org/10.1038/nm1240
PMID:15852016

146. Samuel MA, Wang H, Siddharthan V, Morrey JD, Diamond MS. Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. Proc Natl Acad Sci U S A. 2007; 104:17140–17145.
https://doi.org/10.1073/pnas.0705837104
PMID:17939996

147. Danta CC. CNS Penetration Ability: A Critical Factor for Drugs in the Treatment of SARS-CoV-2 Brain Infection. ACS Chem Neurosci. 2020; 11:2137–2144.
https://doi.org/10.1021/acschemneuro.0c00335
PMID:32639711

148. Jacomy H, Fragoso G, Almazan G, Mushynski WE, Talbot PJ. Human coronavirus OC43 infection induces chronic encephalitis leading to disabilities in BALB/C mice. Virology. 2006; 349:335–346.
https://doi.org/10.1016/j.virol.2006.01.049
PMID:16527322

149. Coraci D, Fusco A, Frizziero A, Giovannini S, Biscotti L, Padua L. Global approaches for global challenges: The possible support of rehabilitation in the management of COVID-19. J Med Virol. 2020; 92:1739–1740.
https://doi.org/10.1002/jmv.25829
PMID:32243596

150. Nath A. Neurologic complications of coronavirus infections. Neurology. 2020; 94:809–810.
https://doi.org/10.1212/wnl.0000000000009455
PMID:32229625

151. Fotuhi M, Mian A, Meysami S, Raji CA. Neurobiology of COVID-19. J Alzheimers Dis. 2020; 76:3–19.
https://doi.org/10.3233/jad-200581
PMID:32538857

Research Paper

The efficiency and safety of high-dose vitamin C in patients with COVID-19: a retrospective cohort study

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ABSTRACT

Background: The inflammatory reaction is the main cause of acute respiratory distress syndrome and multiple organ failure in patients with Coronavirus disease 2019, especially those with severe and critical illness. Several studies suggested that high-dose vitamin C reduced inflammatory reaction associated with sepsis and acute respiratory distress syndrome. This study aimed to determine the efficacy and safety of high-dose vitamin C in Coronavirus disease 2019.

Methods: We included 76 patients with Coronavirus disease 2019, classified into the high-dose vitamin C group (loading dose of 6g intravenous infusion per 12 hr on the first day, and 6g once for the following 4 days, n=46) and the standard therapy group (standard therapy alone, n=30).

Results: The risk of 28-day mortality was reduced for the high-dose vitamin C versus the standard therapy group (HR=0.14, 95% CI, 0.03-0.72). Oxygen support status was improved more with high-dose vitamin C than standard therapy (63.9% vs 36.1%). No safety events were associated with high-dose vitamin C therapy.

Conclusion: High-dose vitamin C may reduce the mortality and improve oxygen support status in patients with Coronavirus disease 2019 without adverse events.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has spread rapidly worldwide [1–5]. As of July 2, 2020, 10,533,779 cases were reported and COVID-19 caused 512,842 death worldwide according to data from the World Health Organization (WHO). More than 50,000 individuals were critically ill [6]. Severe and critically ill patients often have dyspnea and/or hypoxemia and can even rapidly progress to acute respiratory distress

syndrome (ARDS), septic shock, and multiple organ failure, resulting in high mortality. The mortality rate ranges from 4% to 28% [7–12].

Previous studies have reported clinical characteristics of patients with COVID-19 [7–9]. Studies showed a rapid and massive production of many cytokines called a cytokine "storm" or inflammatory "storm" in confirmed COVID-19 patients [7–9]. The production of oxygen free radicals leads to microvascular endothelial injury and increased permeability of the microvasculature, resulting in increased exudation, which may be important causes of ARDS and multiple organ failure.

Physicians and biologists all over the world have been looking for drugs for COVID-19. However, no effective antiviral therapy or vaccine has been confirmed. Recently, several clinical trials have investigated therapeutic drugs, [13–19] but their effectiveness and safety are still controversial.

Early studies [20, 21] reported that the application of vitamin C in animal models of sepsis could improve capillary circulation, microvascular barrier function and arteriolar reactivity caused by vasoconstrictors. As a tissue antioxidant, vitamin C can effectively remove oxygen free radicals produced by myocardial tissues, macrophages and ischemia-reperfusion tissues. These free radicals are the initiating factors of Keshan disease [22, 23]. Physicians in our hospital successfully used high-dose vitamin C with patients with acute Keshan disease and cardiogenic shock and reduced the mortality from 86% to 5% [24–26]. In recent years, physicians have used vitamin C to treat various serious inflammatory diseases, especially ARDS and sepsis [27]. In addition, the high-dose vitamin C therapy in acute Keshan disease [24–26] and recent studies [28–30] suggested that a early and short course from 3 to 5 days of high-dose vitamin C treatment could blocked the inflammatory reaction effectively. Observational studies suggested that nearly 40% of sepsis patients showed vitamin C deficiency, [27, 31] and the concentration of vitamin C in plasma of patients with early sepsis was inversely correlated with multiple organ dysfunction indicators [32]. A study of 167 patients with sepsis and ARDS suggested that mortality and intensive care unit stay were significantly reduced in the high-dose vitamin C group [28].

The clinical application of high-dose vitamin C is expected to improve the prognosis of patients by the production of powerful antioxidant free radicals and inhibition of vascular inflammatory exudation. Therefore, we explored the outcomes in patients hospitalized for COVID-19 who received high-dose vitamin C or standard therapy to demonstrate the efficiency and safety of high-dose vitamin C.

RESULTS

The exclusion of participants

Overall, 84 patients received high-dose vitamin C or standard therapy, but 8 were excluded because they were pregnant (n=2), lactating (n=1), had missing baseline information (n=1), received fewer than 5 days of high-dose vitamin C (n=3), or died within 24 hr (n=1).

Baseline characteristics

Table 1 shows the baseline characteristics of 76 patients: 46 with high-dose vitamin C and 30 standard therapy alone. A total of 48 (63.2%) patients had a diagnosis of moderate COVID-19, and 28 (36.8%) severe or critical disease. The median age was 61 years (IQR, 52 to 71), and the median duration of symptoms before therapy 12 days (IQR, 8 to 16). No patient received invasive mechanical ventilation at baseline; 40 (52.6%) patients received high-flow oxygen or noninvasive positive pressure ventilation. The two therapy groups did not differ in baseline characteristics including laboratory data (Table 1, Supplementary Table 1).

Mortality after high-dose vitamin C therapy and standard therapy

Six (7.9%) patients with severe or critical disease died at the end of 28 days; one (16.7%) received high-dose vitamin C, and 5 (83.3%) standard therapy. On Kaplan-Meier analysis, the risk of mortality was significantly reduced with high-dose vitamin C than standard therapy (HR=0.14, 95% CI, 0.03-0.72) (Figure 1). In patients with severe or critical disease and age > 60, the risk of mortality was lower for patients with high-dose vitamin C than that with standard therapy (HR= 9.91, 95% CI, 1.82-54.00; HR=7.98, 95% CI, 1.24-51.22) (Figure 2A, 2B).

Oxygen support status after high-dose vitamin C therapy and standard therapy

Over a median retrospective time of 18 days (IQR, 10 to 28), 36 (47.4%) patients showed an improvement in oxygen support status, 23 (63.9%) in the high-dose vitamin C group and 13 (36.1%) in the standard therapy group (Table 2). For moderate cases (n=48), oxygen support status was improved for 28 patients, and 17 (60.7%) of them were in the high-dose vitamin C group and 11 (39.3%) in the standard therapy group. For patients with severe or critical disease (n=28), there were 8 patients who showed an improvement, and 6 (75.0%) in the high-dose vitamin C group and 2

Table 1. Clinical characteristics of patients at baseline.

Characteristic	Total (n=76)	High-dose VitC (n=46)	Standard therapy (n=30)	*P* value
Disease severity — no. (%)				0.609
Moderate	48 (63.2)	28 (60.9)	20 (66.6)	
Severe or critical	28 (36.8)	18 (39.1)	10 (33.4)	
Age, median (IQR) — years	61 (52-71)	63 (54-71)	57 (49-67)	0.239
Male sex — no. (%)	35 (46.1)	21 (45.7)	14 (46.7)	0.931
Smoking history— no. (%)	8 (10.5)	5 (10.9)	3 (10.0)	0.904
Coexisting condition — no. (%)				
Diabetes	15 (19.7)	11 (23.9)	4 (13.3)	0.257
Hypertension	22 (28.9)	16 (34.8)	6 (20.0)	0.165
Coronary heart disease	5 (6.6)	3 (6.5)	2 (6.7)	0.980
Underlying lung disease	6 (7.9)	4 (8.7)	2 (6.7)	0.748
Chronic liver disease	4 (5.3)	3 (6.5)	1 (3.3)	0.543
Chronic kidney disease	2 (2.6)	2 (4.3)	0	0.247
Systolic blood pressure, median (IQR) — mmHg	130 (112-141)	127 (112-139)	132 (121-144)	0.532
Duration of symptoms before therapy, median (IQR) — days	12 (8-16)	13 (8-20)	10 (8-12)	0.180
Oxygen support category — no. (%)				0.921
Low-flow oxygen	36 (47.4)	22 (47.8)	14 (46.7)	
High-flow oxygen or noninvasive positive pressure ventilation	40 (52.6)	24 (52.2)	16 (53.3)	
Treatment				
Antiviral therapy	72 (94.7)	42 (91.3)	30 (100.0)	0.097
Antibiotic therapy	70 (92.1)	43 (93.5)	27 (90.0)	0.583
Corticosteroids	28 (36.8)	15 (32.6)	13 (43.3)	0.343
Gamma globulin	23 (30.3)	13 (28.3)	10 (33.3)	0.638
Statins	14 (18.4)	10 (21.7)	4 (13.3)	0.355

IQR: interquartile range, VitC: vitamin C.

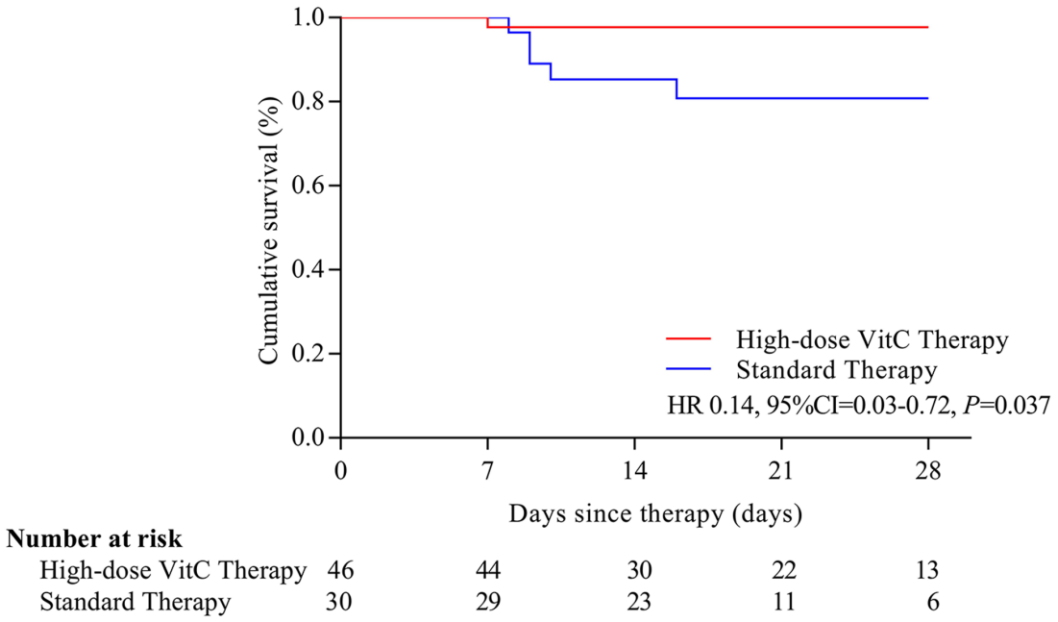

Figure 1. Overall survival with the two treatments in COVID-19 patients. The risk of mortality was significantly reduced with high-dose vitamin C than standard therapy (HR=0.14, 95% CI, 0.03-0.72). VitC: vitamin C.

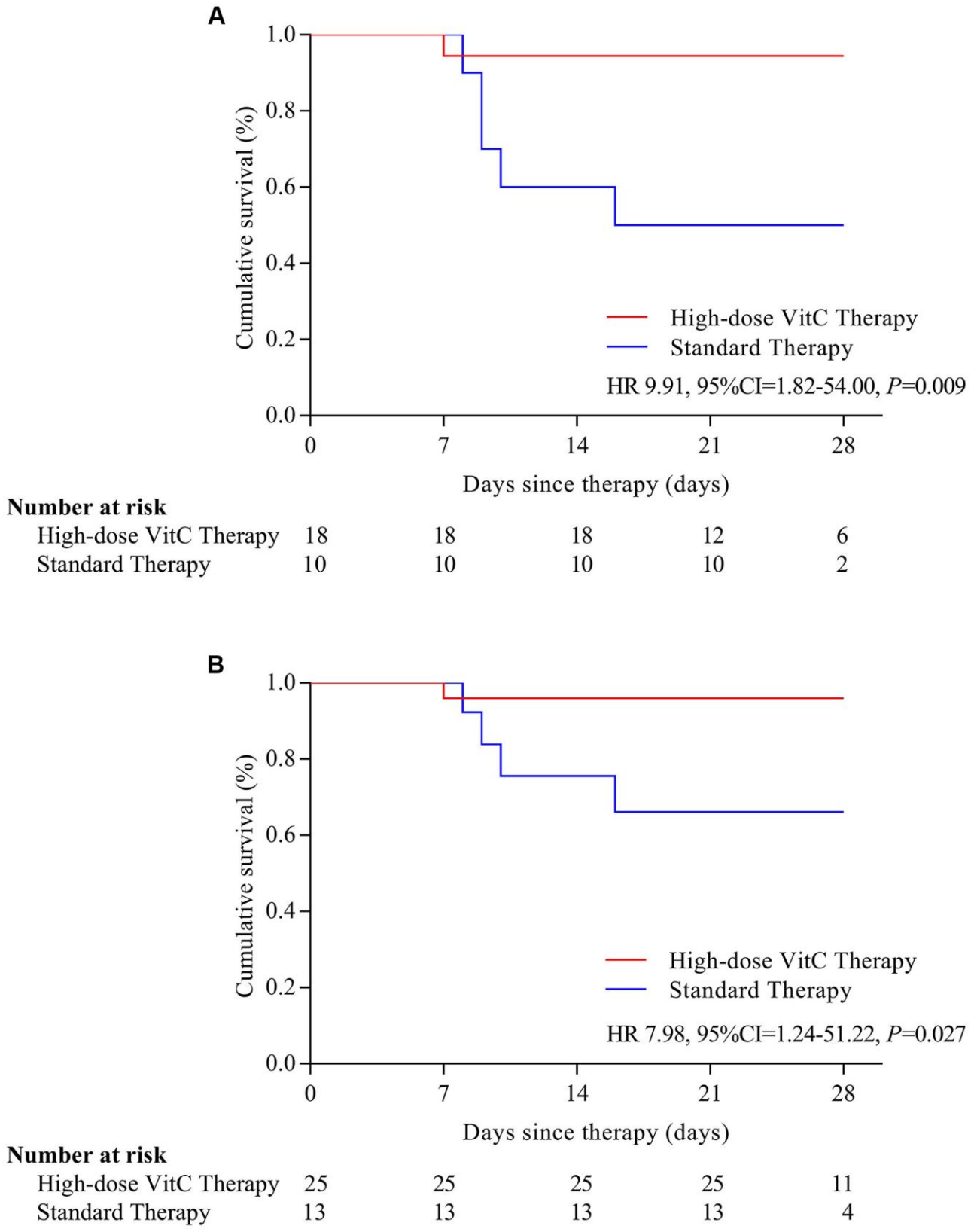

Figure 2. Survival by severe and critical disease (**A**) and age > 60 years old (**B**). Survival is stratified by disease severity at baseline and by age. (**A**) In patients with severe or critical disease, the risk of mortality was lower for patients with high-dose vitamin C than that with standard therapy (HR= 9.91, 95% CI, 1.82-54.00); (**B**) In patients with age > 60, the risk of mortality was lower for patients with high-dose vitamin C than that with standard therapy (HR=7.98, 95% CI, 1.24-51.22). VitC: vitamin C.

Table 2. Changes in oxygen-support status after treatment by disease type.

Oxygen support category	Total (n=76)	High-dose VitC			Standard therapy		
		Total (n=46)	Moderate (n=28)	Severe or critical (n=18)	Total (n=30)	Moderate (n=20)	Severe or critical (n=10)
Low-flow oxygen — no. (%)	14 (18.4)	7 (15.2)	5 (17.9)	2 (11.1)	7 (23.3)	5 (25.0)	2 (20.0)
High-flow oxygen — no. (%)	22 (28.9)	15 (32.6)	7 (25.0)	8 (44.4)	7 (23.3)	6 (30.0)	1 (10.0)
Noninvasive positive pressure ventilation — no. (%)	3 (3.9)	2 (4.3)	0	2 (11.1)	1 (3.3)	0	1 (10.0)
Discharge — no. (%)	31 (40.8)	21 (45.7)	16 (57.1)	5 (27.8)	10 (33.3)	9 (45.0)	1 (10.0)
Death — no. (%)	6 (7.9)	1 (2.2)	0	1 (5.6)	5 (16.7)	0	5 (50.0)
Improvement —no. (%)	36 (47.4)	23 (50.0)	17 (60.7)	6 (33.3)	13 (43.3)	11 (55.0)	2 (20.0)

Percentages of each oxygen support category were calculated with the number of patients at baseline as the denominator. VitC: vitamin C.

(25.0%) in the standard therapy group (Table 2). Moreover, 31 patients were discharged at the end of day 18, 21 (67.7%) in the high-dose vitamin C group, and 10 (32.3%) in the standard therapy group.

The subgroups benefit from high-dose vitamin C therapy

In the high-dose vitamin C group, clinical improvement was better for patients ≤ 60 years old than others (HR=0.49, 95%CI, 0.25-0.99, Supplementary Figure 1A). Moreover, clinical improvement was better for patients who received low-flow oxygen (HR=0.41, 95%CI, 0.20-0.84, Supplementary Figure 1B), and those with serum high-sensitivity C-reactive protein (hs-CRP) < 1 mg/L (HR=0.26, 95%CI, 0.07-0.94, Supplementary Figure 1C) than their counterparts.

Changes in biomarkers of inflammation after high-dose vitamin C therapy and standard therapy

As compared with standard therapy, high-dose vitamin C reduced serum hs-CRP, procalcitonin (PCT) and interleukin-8 (IL-8) levels (Figure 3A, 3B, 3E). The serum interleukin-2 receptor (IL-2R), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) levels were not affected remarkably in the high-dose vitamin C group (Figure 3C, 3D, 3F).

Safety

In total, 19 (41.3%) patients in the high-dose vitamin C group, and 18 (60.0%) in the standard therapy group showed adverse events (Table 3). Thrombocytopenia and increased total bilirubin events were common in the 2 groups. However, the incidence was lower in the high-dose vitamin C than the standard therapy group (8.7% vs 13.3%, 13.0% vs 30.0%). 6 (7.9%) patients showed serious adverse events (respiratory failure or ARDS, shock and sepsis): 1 received high-dose vitamin C and 5 standard therapy. Moreover, respiratory failure or ARDS were more common in the standard therapy than the high-dose vitamin C group.

DISCUSSION

The world is currently facing the threat of the COVID-19 pandemic caused by SARS-CoV-2 infection. This epidemic continues to spread, and there are no vaccines or specific drugs approved or used to prevent or treat COVID-19. A large number of studies have confirmed that high-dose vitamin C can benefit patients with lung injury caused by various inflammatory diseases, especially ARDS and sepsis [27, 30, 33, 34]. This retrospective cohort study analyzed the efficiency and safety of high-dose vitamin C in patients with COVID-19. High-dose vitamin C could decrease mortality and improve the oxygen support status of COVID-19 patients.

Additionally, high-dose vitamin C remarkably reduced serum hs-CRP and PCT levels in COVID-19 patients (Figure 3A, 3B). CRP and PCT are acute-phase inflammatory proteins and related to the severity of body systematic infection. High CRP and PCT levels are associated with organ failure and increased mortality in patients admitted to intensive care units [35, 36]. A study [37] reported lower mortality in COVID-19 patients with reduced CRP level than persistently high CRP level. Jensen and colleagues [35] also found high PCT level as an early independent predictor of mortality for patients admitted to an intensive care unit. Recent reports suggested that COVID-19 patients had high levels of hs-CRP and PCT [36, 38]. Vitamin C can directly reduce the production of reactive oxygen species, maintain endothelial barrier function and vasodilation, and downregulate the expression of proinflammatory modulators [39, 40]. Moreover, high-dose vitamin C

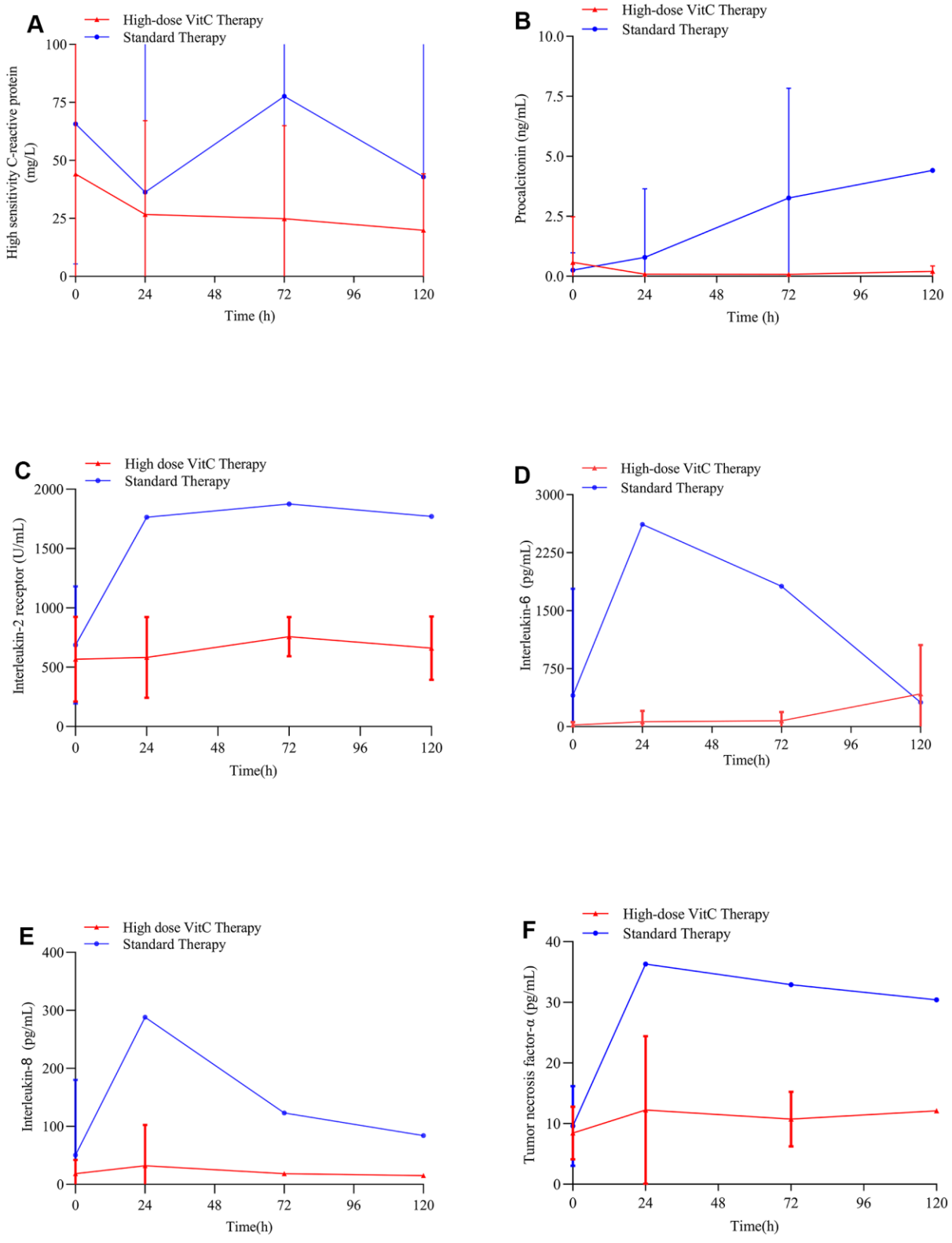

Figure 3. Changes in hs-CRP and PCT levels with therapy. (**A**) High-dose vitamin C reduced serum hs-CRP levels in COVID-19 patients; (**B**) High-dose vitamin C reduced serum PCT levels in COVID-19 patients; (**C**) High-dose vitamin C did not affect the serum IL-2R levels in COVID-19 patients remarkably; (**D**) High-dose vitamin C did not affect the serum IL-6 levels in COVID-19 patients remarkably; (**E**) High-dose vitamin C reduced serum IL-8 levels in COVID-19 patients; (**F**) High-dose vitamin C did not affect the serum TNF-α in COVID-19 patients remarkably. VitC: vitamin C; hs-CRP: high-sensitivity C-reactive protein; PCT: procalcitonin; IL-2R: interleukin-2 receptor; IL-6: interleukin-6; IL-8: interleukin-8; TNF-α: tumor necrosis factor-α levels.

Table 3. Summary of adverse events.

Event	Total (n=76)	High-dose VitC (n=46)	Standard therapy (n=30)
Any adverse event	37 (48.7)	19 (41.3)	18 (60.0)
Lymphopenia	7 (9.2)	3 (6.5)	4 (13.3)
Leukopenia	1 (1.3)	1 (2.2)	0
Thrombocytopenia	8 (10.5)	4 (8.7)	4 (13.3)
Increased aspartate aminotransferase activity	5 (6.6)	3 (6.5)	2 (6.7)
Increased alanine aminotransferase activity	3 (3.9)	2 (4.3)	1 (3.3)
Increased total bilirubin level	15 (19.7)	6 (13.0)	9 (30.0)
Increased serum creatinine level	6 (7.9)	3 (6.5)	3 (10.0)
Increased creatine kinase isoenzyme-MB activity	2 (2.6)	1 (2.2)	1 (3.3)
Increased high sensitivity-cardiac troponin I level	5 (6.6)	3 (6.5)	2 (6.7)
Increased N-terminal pro-B-type natriuretic peptide level	5 (6.6)	3 (6.5)	2 (6.7)
Serious adverse event	6 (7.9)	1 (2.2)	5 (16.7)
Respiratory failure or ARDS	5 (6.6)	1 (2.2)	4 (13.3)
Shock	2 (2.6)	1 (2.2)	1 (3.3)
Sepsis	2 (2.6)	1 (2.2)	1 (3.3)

Data are n (%). Adverse events that occurred in more than 1 patient through follow-up. Some patients had more than one adverse event. All deaths were caused by respiratory failure. ARDS: acute respiratory distress syndrome; VitC: vitamin C.

reduced serum IL-8 level compared with the standard therapy in COVID-19 patients (Figure 3E). The synergy of cytokines such as TNF-α, interleukin-1 (IL-1), IL-6, and IL-8 can regulate the inflammatory cascade. Studies have shown that vitamin C reduced the production of chemokine such as IL-8, thereby reducing the inflammatory changes of lung injury caused by sepsis, and this reaction was associated with significantly lower mortality in critically ill patients with severe pneumonia [30, 34]. Evidence has suggested that cytokine storms such as higher concentrations of IL-6, IL-8 and interleukin-10 (IL-10) were related to the prognosis of COVID-19 patients [41]. Treatment with high-dose vitamin C is associated with reduced hs-CRP, PCT, and IL-8 levels and can reduce inflammation and thus attenuate lung and systemic inflammation [30, 33, 34]. Also, our COVID-19 patients showed improved oxygen support status, which may lead to reduced mortality.

Epidemiology results showed that patients with severe and critical disease have relatively high mortality, and no effective treatment is available to significantly reduce mortality [42, 43]. Our study found that high-dose vitamin C could significantly decrease mortality with COVID-19 as compared with standard therapy, so vitamin C may be an effective drug for COVID-19, especially for severe and critical cases.

Additionally, we found better clinical improvement with high-dose vitamin C therapy than standard therapy for patients ≤ 60 years old and with low-flow oxygen, and serum hs-CRP level < 1 mg/L. Thus, the use of high-dose vitamin C may have a certain subgroup selectivity, although the clinical improvement between high-dose vitamin C and standard therapy did not significantly differ (Supplementary Figure 2). Body weight is an important clinical indicator for many diseases. While many previous studies [11, 44, 45] have confirmed that the body weight was not associated with the prognosis of patients with COVID-19. In addition, the effect of high-dose vitamin C on anti-oxidation and reducing inflammatory reaction did not show significant correlation with body weight based on recent studies [30, 46]. Although we did not record the body weight of patients in baseline, the body weight might not affect the results of our study.

The incidence of adverse events was lower with high-dose vitamin C than standard therapy, without new adverse events, which suggests that high-dose vitamin C may be safe. Moreover, many studies have confirmed the safety of high-dose vitamin C. A study of high-dose vitamin C treatment in patients with severe sepsis and ARDS found no adverse events [31, 43]. Recent studies [8, 47, 48] reported that some patients with COVID-19 had lymphopenia, leukopenia, thrombocytopenia, liver function abnormality, abnormal myocardial zymography findings, and renal impairment. Therefore, the adverse events in our study were not associated with high-dose vitamin C therapy.

Physicians are exploring various drugs for COVID-19. Remdesivir, [13, 14] hydroxychloroquine/chloroquine [15–17] and corticosteroids [49] have been explored. Although most achieved a certain effect, they were accompanied by various adverse events such as arrhythmia, [18, 19] immunosuppressive effects, [49] liver and kidney damage, [13] and acute respiratory failure [13, 14]. Generally, serious adverse events have a great impact on the treatment effect, prognosis and quality of life of patients. High-dose vitamin C with an effective role and without adverse events might be a promising therapy for COVID-19.

This study has several limitations. First, although we did not observe differences at baseline between the two treatment groups, potential bias exists in this retrospective cohort study. For instance, the treatment patients received and the specific protocols of standard therapy were not controlled. Second, the results are less persuasive owing to the small sample size. Third, we did not collect data on plasma vitamin C level to confirm whether the plasma level is a predictor in patients with COVID-19.

CONCLUSIONS

In summary, high-dose vitamin C may reduce inflammatory reaction, improve oxygen support status and reduce mortality in COVID-19 patients, without adverse events. Also, it may be effective for certain subgroups with severe and critical disease and older patients. High-dose vitamin C may be a promising therapy for COVID-19.

MATERIALS AND METHODS

Study design and participants

This was a retrospective cohort study of in-hospital patients with COVID-19 from January 31, 2020 to March 28, 2020 diagnosed and treated by our medical group in Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. This study was based on the approved guidelines for COVID-19 [50]. Informed consent was waived by the board due to the retrospective nature of the study.

Patients with COVID-19 were diagnosed according to Diagnosis and Treatment of Pneumonia Infected by Novel Coronavirus issued by the National Health Commission of China [51]. We excluded patients who were younger than 18 years, allergic to vitamin C, died within 24 hr after admission, or were pregnant and/or lactating. Other patients were classified into two groups: high-dose vitamin C therapy and standard therapy. Patients were informed of the rationality of the treatment plan and potential side effects. Only patients with consent were treated. Patients in the high-dose vitamin C therapy group received standard therapy [51] as well as a 5-day course of a loading dose of 6g added to 5% glucose solution for intravenous high-dose vitamin C infusion lasting over 60 min per 12 hr on the first day, plus 6g added to 5% glucose solution for intravenous high-dose vitamin C infusion lasting over 60 min per day for the following 4 days. Patients receiving standard therapy alone were included in the standard therapy group. The standard therapy was based on the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia issued by the National Health Commission of China [51]. As suggested in the guideline, standard therapy included daily monitoring, routine laboratory tests (blood count, urea, creatinine, liver enzymes, and other related biomarkers), effective respiratory therapy (nasal cannula oxygen therapy, mask oxygen therapy or high-flow nasal oxygen therapy, if necessary, noninvasive ventilation), and surveillance of vital parameters according to the patient's condition. The addition of other therapies such as antibiotics, corticosteroids, immunomodulators and other antivirals (e.g., Lopinavir/Ritonavir, Ribavirin) according to the assessment of the physicians were also included in the standard therapy.

This study lasted from the time of hospital admission to discharge or death in hospital occurred. The hospital electronic medical records included the data of all patients from admission to discharge or death in hospital, the data during treatment with high-dose vitamin C was also included. In addition, the 28-day death of patients was recorded.

Outcomes

The primary outcome was 28-day mortality and clinical improvement. The secondary outcome was change in oxygen support status after treatment. Clinical improvement was defined as a decrease of at least 2 points from baseline to day 28 or discharge according to the seven-category ordinal scale. The ordinal scale is based on the endpoints in patients with severe influenza, [51–53] which consists of 1) no hospitalization with the resumption of normal activities; 2) no hospitalization but unable to resume normal activities; 3) hospitalization but not requiring supplemental oxygen; 4) hospitalization and requiring supplemental oxygen; 5) hospitalization and requiring nasal high-flow oxygen therapy and/or non-invasive mechanical ventilation; 6) hospitalization and requiring extracorporeal membrane oxygenation and/or invasive mechanical ventilation; and 7) death.

Statistical analysis

Information for patients including clinical characteristics, laboratory data and outcomes were

collected from medical records. Continuous variables for baseline characteristics are described with the median (interquartile range [IQR]) and categorical variables with frequency (percentage). Continuous variables were analyzed using Student's *t* or Mann-Whitney *U* tests and categorical variables were analyzed with chi-squared or Fisher' s exact tests. Clinical improvement and all-cause mortality were analyzed by Kaplan-Meier analysis with a log-rank test. Hazard ratios and 95% confidence intervals (CIs) were estimated. Data were analyzed by using SPSS 20.0. $P<0.05$ was considered statistically significant.

Abbreviations

COVID-19: Coronavirus disease 2019; WHO: World Health Organization; ARDS: acute respiratory distress syndrome; IQR: interquartile range; CIs: confidence intervals; hs-CRP: high-sensitivity C-reactive protein; PCT: procalcitonin; IL-1: interleukin-1; IL-2R: interleukin-2 receptor; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α.

AUTHOR CONTRIBUTIONS

Conceptualization, Gang Wang; Methodology, Jianrui Lv and Xiaorong Ma; Software, Yonghong Guo and Dexin Zhang; Validation, Huiyun Yang and Wei Jiang; Formal Analysis, Dengfeng Gao and Min Xu; Investigation, Fuxue Deng; Resources, Guozhi Xia; Data Curation, Ziwei Lu and Lv Lv; Writing – Original Draft Preparation, Min Xu; Writing – Review and Editing, Dengfeng Gao and Shouping Gong; Visualization, Min Xu and Lv Lv; Supervision, Dengfeng Gao and Shouping Gong; Project Administration, Shouping Gong.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33. https://doi.org/10.1056/NEJMoa2001017 PMID:31978945

2. Lu H, Stratton CW, Tang YW. Outbreak of pneumonia of unknown etiology in Wuhan, China: the mystery and the miracle. J Med Virol. 2020; 92:401–02. https://doi.org/10.1002/jmv.25678 PMID:31950516

3. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. Lancet. 2020; 395:470–73. https://doi.org/10.1016/S0140-6736(20)30185-9 PMID:31986257

4. Wuhan Municipal Health Commission. Report of novel coronavirus-infected pneumonia in China (in Chinese). 2020. http://wjw.wuhan.gov.cn

5. Hui DS, I Azhar E, Madani TA, Ntoumi F, Kock R, Dar O, Ippolito G, Mchugh TD, Memish ZA, Drosten C, Zumla A, Petersen E. The continuing 2019-nCoV epidemic threat of novel coronaviruses to global health - the latest 2019 novel coronavirus outbreak in Wuhan, China. Int J Infect Dis. 2020; 91:264–66. https://doi.org/10.1016/j.ijid.2020.01.009 PMID:31953166

6. World Health Organization. Coronavirus disease (COVID-19) panndemic. 2020. https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200702-covid-19-sitrep-164.pdf?sfvrsn=ac074f58_2

7. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

8. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

9. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, Wang B, Xiang H, Cheng Z, Xiong Y, Zhao Y, Li Y, Wang X, Peng Z. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus-infected pneumonia in Wuhan, China. JAMA. 2020; 323:1061–69. https://doi.org/10.1001/jama.2020.1585 PMID:32031570

10. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091.

https://doi.org/10.1136/bmj.m1091
PMID:32217556

11. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

12. Richardson S, Hirsch JS, Narasimhan M, Crawford JM, McGinn T, Davidson KW, Barnaby DP, Becker LB, Chelico JD, Cohen SL, Cookingham J, Coppa K, Diefenbach MA, et al, and the Northwell COVID-19 Research Consortium. Presenting characteristics, comorbidities, and outcomes among 5700 patients hospitalized with COVID-19 in the New York city area. JAMA. 2020; 323:2052–59. https://doi.org/10.1001/jama.2020.6775 PMID:32320003

13. Goldman JD, Lye DC, Hui DS, Marks KM, Bruno R, Montejano R, Spinner CD, Galli M, Ahn MY, Nahass RG, Chen YS, SenGupta D, Hyland RH, et al, and GS-US-540-5773 Investigators. Remdesivir for 5 or 10 days in patients with severe covid-19. N Engl J Med. 2020; 383:1827–37. https://doi.org/10.1056/NEJMoa2015301 PMID:32459919

14. Wang Y, Zhang D, Du G, Du R, Zhao J, Jin Y, Fu S, Gao L, Cheng Z, Lu Q, Hu Y, Luo G, Wang K, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet. 2020; 395:1569–78. https://doi.org/10.1016/S0140-6736(20)31022-9 PMID:32423584

15. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 2020; 30:269–71. https://doi.org/10.1038/s41422-020-0282-0 PMID:32020029

16. Chen Z, Hu J, Zhang Z, Jiang S, Han S, Yan D, Zhuang R, Hu B, Zhang Z. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. medRxiv. 2020. https://doi.org/10.1101/2020.03.22.20040758

17. Boulware DR, Pullen MF, Bangdiwala AS, Pastick KA, Lofgren SM, Okafor EC, Skipper CP, Nascene AA, Nicol MR, Abassi M, Engen NW, Cheng MP, LaBar D, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for covid-19. N Engl J Med. 2020; 383:517–25. https://doi.org/10.1056/NEJMoa2016638 PMID:32492293

18. Borba MG, Val FF, Sampaio VS, Alexandre MA, Melo GC, Brito M, Mourão MP, Brito-Sousa JD, Baía-da-Silva D, Guerra MV, Hajjar LA, Pinto RC, Balieiro AA, et al, and CloroCovid-19 Team. Effect of high vs low doses of chloroquine diphosphate as adjunctive therapy for patients hospitalized with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection: a randomized clinical trial. JAMA Netw Open. 2020; 3:e208857. https://doi.org/10.1001/jamanetworkopen.2020.8857 PMID:32330277

19. Chorin E, Dai M, Shulman E, Wadhwani L, Bar-Cohen R, Barbhaiya C, Aizer A, Holmes D, Bernstein S, Spinelli M, Park DS, Chinitz LA, Jankelson L. The QT interval in patients with COVID-19 treated with hydroxychloroquine and azithromycin. Nat Med. 2020; 26:808–09. https://doi.org/10.1038/s41591-020-0888-2 PMID:32488217

20. Armour J, Tyml K, Lidington D, Wilson JX. Ascorbate prevents microvascular dysfunction in the skeletal muscle of the septic rat. J Appl Physiol (1985). 2001; 90:795–803. https://doi.org/10.1152/jappl.2001.90.3.795 PMID:11181585

21. Wu F, Wilson JX, Tyml K. Ascorbate protects against impaired arteriolar constriction in sepsis by inhibiting inducible nitric oxide synthase expression. Free Radic Biol Med. 2004; 37:1282–89. https://doi.org/10.1016/j.freeradbiomed.2004.06.025 PMID:15451067

22. Chakrabarty S, Nandi A, Mukhopadhyay CK, Chatterjee IB. Protective role of ascorbic acid against lipid peroxidation and myocardial injury. Mol Cell Biochem. 1992; 111:41–47. https://doi.org/10.1007/BF00229572 PMID:1588941

23. Mukhopadhyay CK, Ghosh MK, Chatterjee IB. Ascorbic acid prevents lipid peroxidation and oxidative damage of proteins in Guinea pig extrahepatic tissue microsomes. Mol Cell Biochem. 1995; 142:71–78. https://doi.org/10.1007/BF00928915 PMID:7753044

24. Li F. Mechanism of rescue of Keshan disease with high-dose vitamin C. Zhongguo Di Fang Bing Fang Zhi Za Zhi. 1996; 11:33–36.

25. Keshan Disease Laboratory of Xi'an Medical College. Clinical effect and mechanism of high-dose vitamin C for severe Keshan disease. Zhonghua Nei Ke Za Zhi. 1961; 9:436.

26. Wang S. Changes in thinking and the establishment of high-dose vitamin C therapy-commemorating the 60th anniversary of the discovery of Keshan disease and the

35th anniversary of the high-dose vitamin C therapy. Di Fang Bing Tong Bao. 1996; 11:7.

27. Marik PE, Khangoora V, Rivera R, Hooper MH, Catravas J. Hydrocortisone, vitamin C, and thiamine for the treatment of severe sepsis and septic shock: a retrospective before-after study. Chest. 2017; 151:1229–38. https://doi.org/10.1016/j.chest.2016.11.036 PMID:27940189

28. Fowler AA 3rd, Truwit JD, Hite RD, Morris PE, DeWilde C, Priday A, Fisher B, Thacker LR 2nd, Natarajan R, Brophy DF, Sculthorpe R, Nanchal R, Syed A, et al. Effect of vitamin C infusion on organ failure and biomarkers of inflammation and vascular injury in patients with sepsis and severe acute respiratory failure: the CITRIS-ALI randomized clinical trial. JAMA. 2019; 322:1261–70. https://doi.org/10.1001/jama.2019.11825 PMID:31573637

29. Fowler AA 3rd, Syed AA, Knowlson S, Sculthorpe R, Farthing D, DeWilde C, Farthing CA, Larus TL, Martin E, Brophy DF, Gupta S, Fisher BJ, Natarajan R, and Medical Respiratory Intensive Care Unit Nursing. Phase I safety trial of intravenous ascorbic acid in patients with severe sepsis. J Transl Med. 2014; 12:32. https://doi.org/10.1186/1479-5876-12-32 PMID:24484547

30. Kim WY, Jo EJ, Eom JS, Mok J, Kim MH, Kim KU, Park HK, Lee MK, Lee K. Combined vitamin C, hydrocortisone, and thiamine therapy for patients with severe pneumonia who were admitted to the intensive care unit: propensity score-based analysis of a before-after cohort study. J Crit Care. 2018; 47:211–18. https://doi.org/10.1016/j.jcrc.2018.07.004 PMID:30029205

31. Carr AC, Rosengrave PC, Bayer S, Chambers S, Mehrtens J, Shaw GM. Hypovitaminosis C and vitamin C deficiency in critically ill patients despite recommended enteral and parenteral intakes. Crit Care. 2017; 21:300. https://doi.org/10.1186/s13054-017-1891-y PMID:29228951

32. Borrelli E, Roux-Lombard P, Grau GE, Girardin E, Ricou B, Dayer J, Suter PM. Plasma concentrations of cytokines, their soluble receptors, and antioxidant vitamins can predict the development of multiple organ failure in patients at risk. Crit Care Med. 1996; 24:392–97. https://doi.org/10.1097/00003246-199603000-00006 PMID:8625625

33. Hemilä H, Louhiala P. Vitamin C for preventing and treating pneumonia. Cochrane Database Syst Rev. 2013; 8:CD005532. https://doi.org/10.1002/14651858.CD005532.pub3 PMID:23925826

34. Fisher BJ, Seropian IM, Kraskauskas D, Thakkar JN, Voelkel NF, Fowler AA 3rd, Natarajan R. Ascorbic acid attenuates lipopolysaccharide-induced acute lung injury. Crit Care Med. 2011; 39:1454–60. https://doi.org/10.1097/CCM.0b013e3182120cb8 PMID:21358394

35. Jensen JU, Heslet L, Jensen TH, Espersen K, Steffensen P, Tvede M. Procalcitonin increase in early identification of critically ill patients at high risk of mortality. Crit Care Med. 2006; 34:2596–602. https://doi.org/10.1097/01.CCM.0000239116.01855.61 PMID:16915118

36. Chen L, Liu HG, Liu W, Liu J, Liu K, Shang J, Deng Y, Wei S. [Analysis of Clinical Features of 29 Patients With 2019 Novel Coronavirus Pneumonia]. Zhonghua Jie He He Hu Xi Za Zhi. 2020; 43:203–08.

37. Lobo SM, Lobo FR, Bota DP, Lopes-Ferreira F, Soliman HM, Mélot C, Vincent JL. C-reactive protein levels correlate with mortality and organ failure in critically ill patients. Chest. 2003; 123:2043–49. https://doi.org/10.1378/chest.123.6.2043 PMID:12796187

38. Vincent JL, Donadello K, Schmit X. Biomarkers in the critically ill patient: C-reactive protein. Crit Care Clin. 2011; 27:241–51. https://doi.org/10.1016/j.ccc.2010.12.010 PMID:21440199

39. Wilson JX. Mechanism of action of vitamin C in sepsis: ascorbate modulates redox signaling in endothelium. Biofactors. 2009; 35:5–13. https://doi.org/10.1002/biof.7 PMID:19319840

40. May JM, Harrison FE. Role of vitamin C in the function of the vascular endothelium. Antioxid Redox Signal. 2013; 19:2068–83. https://doi.org/10.1089/ars.2013.5205 PMID:23581713

41. Li Q, Xu W, Li WX, Huang CL, Chen L. Dynamics of cytokines and lymphocyte subsets associated with the poor prognosis of severe COVID-19. Eur Rev Med Pharmacol Sci. 2020; 24:12536–44. https://doi.org/10.26355/eurrev_202012_24051 PMID:33336774

42. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

43. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

44. Mikami T, Miyashita H, Yamada T, Harrington M, Steinberg D, Dunn A, Siau E. Risk factors for mortality in patients with COVID-19 in New York city. J Gen Intern Med. 2020; 136:17-26. https://doi.org/10.1007/s11606-020-05983-z PMID:32607928

45. Du RH, Liang LR, Yang CQ, Wang W, Cao TZ, Li M, Guo GY, Du J, Zheng CL, Zhu Q, Hu M, Li XY, Peng P, Shi HZ. Predictors of mortality for patients with COVID-19 pneumonia caused by SARS-CoV-2: a prospective cohort study. Eur Respir J. 2020; 55:2000524. https://doi.org/10.1183/13993003.00524-2020 PMID:32269088

46. Ahn JH, Oh DK, Huh JW, Lim CM, Koh Y, Hong SB. Vitamin C alone does not improve treatment outcomes in mechanically ventilated patients with severe sepsis or septic shock: a retrospective cohort study. J Thorac Dis. 2019; 11:1562–70. https://doi.org/10.21037/jtd.2019.03.03 PMID:31179100

47. Xu XW, Wu XX, Jiang XG, Xu KJ, Ying LJ, Ma CL, Li SB, Wang HY, Zhang S, Gao HN, Sheng JF, Cai HL, Qiu YQ, Li LJ. Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series. BMJ. 2020; 368:m606. https://doi.org/10.1136/bmj.m606 PMID:32075786

48. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, Spitters C, Ericson K, Wilkerson S, Tural A, Diaz G, Cohn A, Fox L, et al, and Washington State 2019-nCoV Case Investigation Team. First case of 2019 novel coronavirus in the United States. N Engl J Med. 2020; 382:929–36. https://doi.org/10.1056/NEJMoa2001191 PMID:32004427

49. Russell CD, Millar JE, Baillie JK. Clinical evidence does not support corticosteroid treatment for 2019-nCoV lung injury. Lancet. 2020; 395:473–75. https://doi.org/10.1016/S0140-6736(20)30317-2 PMID:32043983

50. Comprehensive treatment and management of corona virus disease 2019: expert consensus statement from Shanghai City. Zhonghua Chuan Ran Bing Za Zhi. 2020; 38:134–38.

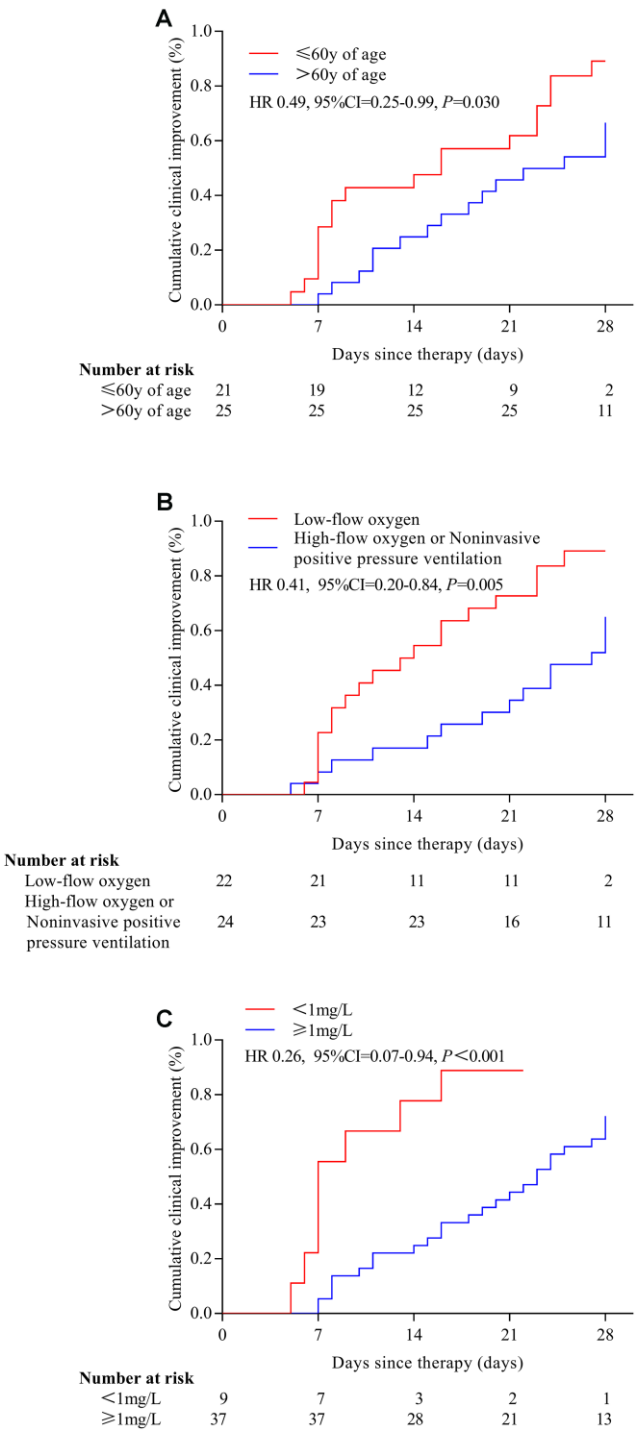
51. Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7). 2020. http://www.gov.cn/zhengce/zhengceku/2020-03/04/content_5486705.htm

52. Wang Y, Fan G, Salam A, Horby P, Hayden FG, Chen C, Pan J, Zheng J, Lu B, Guo L, Wang C, Cao B. Comparative effectiveness of combined favipiravir and oseltamivir therapy versus oseltamivir monotherapy in critically ill patients with influenza virus infection. J Infect Dis. 2020; 221:1688–98. https://doi.org/10.1093/infdis/jiz656 PMID:31822885

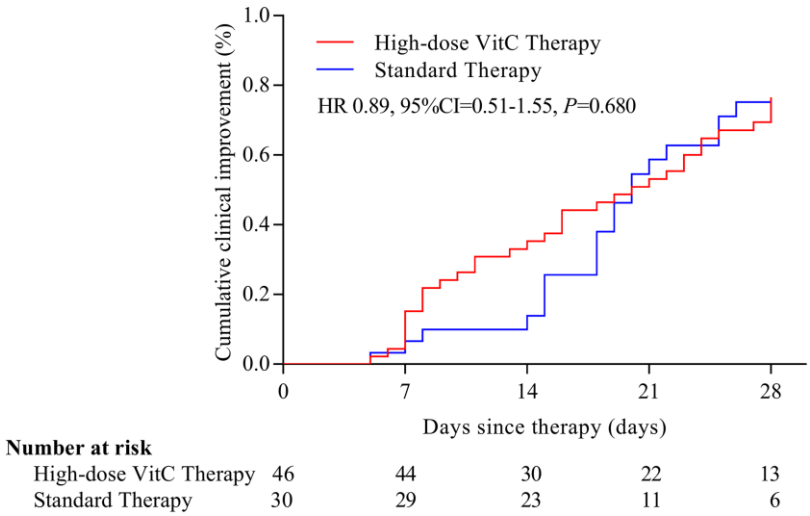
53. Beigel JH, Tebas P, Elie-Turenne MC, Bajwa E, Bell TE, Cairns CB, Shoham S, Deville JG, Feucht E, Feinberg J, Luke T, Raviprakash K, Danko J, et al, and IRC002 Study Team. Immune plasma for the treatment of severe influenza: an open-label, multicentre, phase 2 randomised study. Lancet Respir Med. 2017; 5:500–11. https://doi.org/10.1016/S2213-2600(17)30174-1 PMID:28522352

SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. Cumulative incidence of clinical improvement in high-dose vitamin C therapy group. (**A**) In the high-dose vitamin C group, clinical improvement was better for patients ≤ 60 years old than others (HR=0.49, 95%CI, 0.25-0.99); (**B**) In the high-dose vitamin C group, clinical improvement was better for patients who received low-flow oxygen (HR=0.41, 95%CI, 0.20-0.84); (**C**) In the high-dose vitamin C group, clinical improvement was better for those with serum hs-CRP < 1 mg/L (HR=0.26, 95%CI, 0.07-0.94). Hs-CRP: high-sensitivity C-reactive protein.



Supplementary Figure 2. Overall cumulative incidence of clinical improvement. The clinical improvement between high-dose vitamin C and standard therapy did not significantly differ (HR=0.89, 95%CI, 0.51-1.55). VitC: vitamin C.

Supplementary Table

Supplementary Table 1. Laboratory data for patients at baseline.

Characteristic	Total (n=76)	High-dose VitC (n=46)	Standard therapy (n=30)	P value
White-cell count (×10−9/L) — median (IQR)	6.7 (4.5-9.1)	6.9 (5.0-9.8)	6.2 (4.4-8.5)	0.276
Lymphocyte count (×10−9/L) — median (IQR)	0.9 (0.7-1.4)	1.0 (0.8-1.6)	0.9 (0.6-1.3)	0.224
Platelet count (×10−9/L) — median (IQR)	210 (168-275)	222 (172-283)	186 (152-231)	0.115
Alanine aminotransferase (U/L) — median (IQR)	22 (14-37)	21 (14-37)	25 (15-38)	0.595
Aspartate aminotransferase (U/L) — median (IQR)	28 (20-43)	26 (17-37)	34 (23-49)	0.059
Serum creatinine (μmol/L) — median (IQR)	67 (60-77)	66 (61-72)	70 (56-83)	0.431
High sensitivity-cardiac troponin I (pg/mL) — median (IQR)	3.5 (2.0-13.6)	3.2 (1.9-15.3)	4.3 (2.3-10.2)	0.765
N-terminal pro-B-type natriuretic peptide (pg/mL) — median (IQR)	113 (47-353)	113 (54-675)	105 (30-217)	0.258
Creatine Kinase Isoenzyme-MB (ng/mL) — median (IQR)	0.9 (0.4-2.2)	0.7 (0.4-2.0)	1.3 (0.3-2.6)	0.754
Lactate dehydrogenase (U/L) — median (IQR)	261 (206-365)	241 (195-356)	309 (229-385)	0.138
High sensitivity C-reactive protein (mg/L) — median (IQR)	9.7 (1.6-76.6)	53.9 (10.0-115.8)	18.7 (3.0-84.2)	0.129
Procalcitonin (ng/mL) — median (IQR)	0.04 (0.02-0.17)	0.07 (0.03-0.11)	0.05 (0.03-0.12)	0.403
Interleukin-2 receptor (U/mL) — median (IQR)	490 (241-899)	630 (356-793)	508 (281-853)	0.353
Interleukin-6 (pg/mL) — median (IQR)	4.70 (1.93-20.02)	3.70(1.78-37.79)	4.69 (1.94-23.49)	0.338
Interleukin-8 (pg/mL) — median (IQR)	11.6 (6.8-20.4)	10.3 (5.8-26.5)	11.3 (6.4-20.4)	0.414
Tumor necrosis factor-α (pg/mL) — median (IQR)	7.3 (5.6-10.1)	8.1 (5.2-10.7)	7.7 (5.6-10.3)	0.472
pO2 (mmHg) — median (IQR)	126.7 (124.3-129.6)	126.7 (124.0-130.9)	126.7 (124.6-128.3)	0.577
pCO2 (mmHg) — median (IQR)	40.1 (39.2-41.0)	40.0 (39.2-41.2)	40.3 (39.3-40.9)	0.992
SO2% (%) — median (IQR)	91.2 (88.7-95.5)	92.9 (88.0-96.0)	90.8 (89.0-92.9)	0.414
TCO2 (mmol/L) — median (IQR)	28.2 (24.5-31.7)	27.9 (23.8-30.6)	29.4 (26.3-32.3)	0.122

IQR: interquartile range; VitC: vitamin C.

Research Paper

Convalescent plasma to treat COVID-19: clinical experience and efficacy

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ABSTRACT

The recent outbreak of COVID-19 in the world is currently a big threat to global health and economy. Convalescent plasma has been confirmed effective against the novel corona virus in preliminary studies. In this paper, we first described the therapeutic schedule, antibody detection method, indications, contraindications of the convalescent plasmas and reported the effectiveness of convalescent plasma therapy by a retrospective cohort study.

INTRODUCTION

In December 2019, pneumonia associated with a novel corona virus 2019 (COVID-19) caused an outbreak, which has posed significant threats to global health and economy [1]. As of 6th April 2020, the World Health Organization Situation Reported that this epidemic had spread to more than 180 countries with 1,113,758 confirmed cases, including 62,784 deaths [2]. It was reported that severely/critically ill case ratio was approximately 7-10% [3], while the current treatment strategy mainly rely on the supportive care since specific drugs of COVID-19 are still being researched. On March 4, 2020, in order to improve the therapeutic effect of COVID-19, the National Health Commission of the People's Republic of China organized Chinese experts to make revisions of the "Clinical treatment of COVID-19 Convalescent Plasma (the second trial edition)" [4]. On March 24, 2020, FDA approved the testing of convalescent plasmas for patients with serious or immediately life-threatening COVID-19 infections [5]. To date, thousands of convalescent plasmas have been collected and remarkable efficacy has been achieved in severely and critically ill COVID-19 patients in China. In order to standardize the treatment of COVID-19 Convalescent Plasma and share the clinical experience with the world, we summarized the therapeutic schedule as follows (Figure 1).

RESULTS

Recruit recovered COVID-19 patients

1. Inclusion Criteria (All six criteria must be met)
   - More than 3 weeks after the onset of symptoms of the COVID-19 and complete resolution of symptoms at least 14 days prior to donation.
   - In accordance with relieved isolation and discharge standards following the latest version of the therapeutic schedule.

- Age: 18 -55 years old.
- Weight: male≥50kg, female≥45kg.
- No history of blood-transmitted diseases.
- Eligible donors must be assessed by clinicians according to treatment.

2. Exclusion Criteria
   - With a history of pregnancy or transfusion whose HNA antibody and HLA antibody are positive.
   - Individual's physical condition is not eligible assessed by clinicians.
3. Verify the identities
4. Sign the informed consent
5. Health inquiry, physical examination, laboratory examination of blood samples (refer to technical operation procedures of blood station).

Collection and preparation of the convalescent plasma

1. Collection
   - Equipment and Operations: fully automatic apheresis machine or a fully automatic blood cell separator (refer to technical operation procedures of blood station).
   - Volume: 200-400ml (The exact volume should be assessed by clinicians).
   - The interval between plasma collection should be more than two weeks.
2. Storage
   - Follow the principle of sterility, repackaging the plasma 100-200ml each.
   - Store at 2-6° C for 48 hours. For long-term storage, it should be rapidly frozen to -20° C.

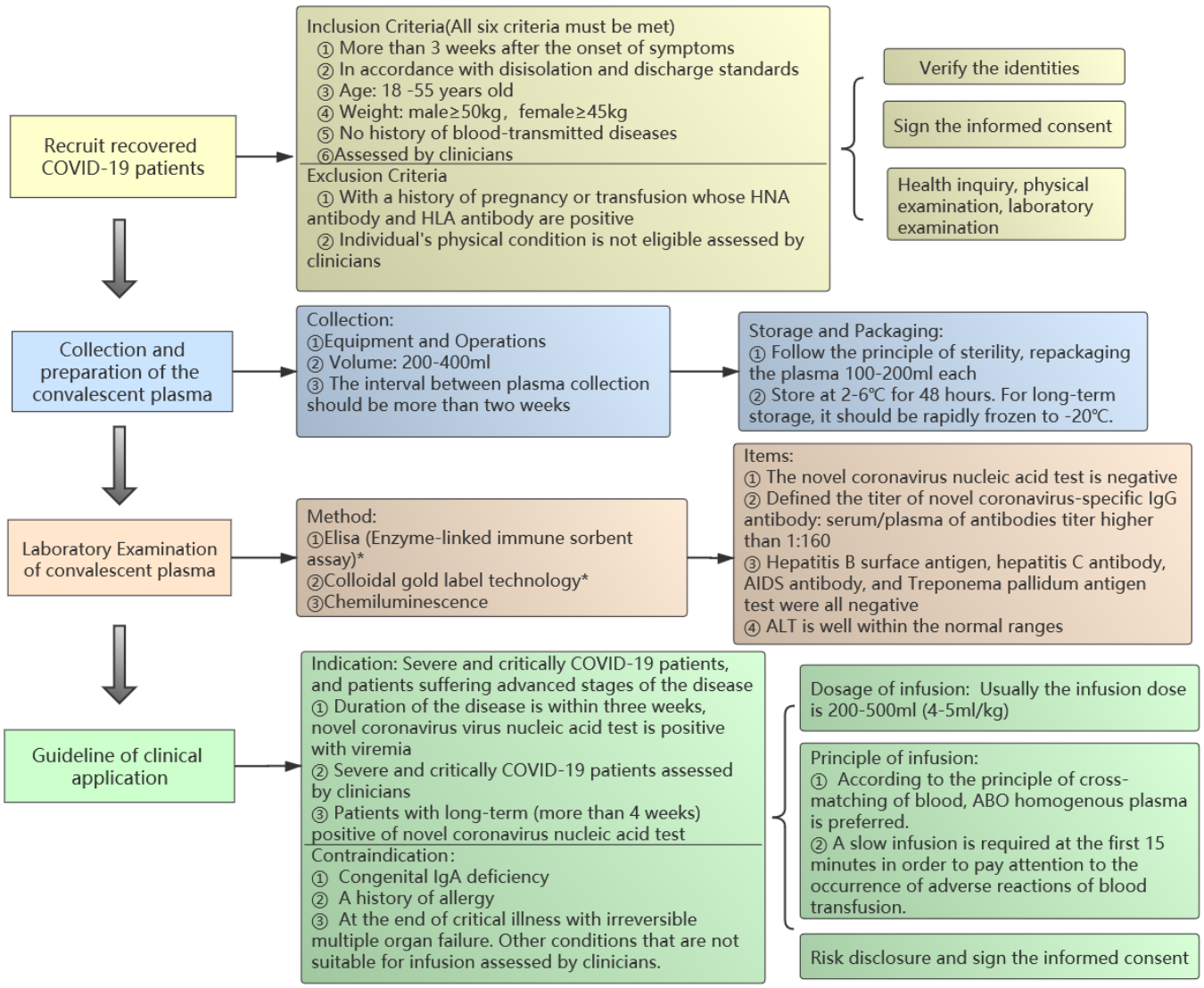

Figure 1. The standardized flow chart of the convalescent plasma transfusion.

3. Packaging: Labelling Requirements: refer to technical operation procedures of blood station.

Laboratory examination of convalescent plasma

1. Method: Elisa (Enzyme-linked immune sorbent assay)*, Colloidal gold label technology*, Chemiluminescence.
   *Note: We recommend using ELISA to detect the novel coronavirus antibody titer since the colloidal gold method was not suitable for titer detection and the false negative rate was high. The analysis of 17 samples showed that the positive rate and sensitivity of ELISA were significantly better than colloidal gold (The specific data is shown in Supplementary Table 1).
2. Items
   - The novel coronavirus nucleic acid test is negative.
   - Defined the titer of novel coronavirus-specific IgG antibody: serum/plasma of antibodies titer higher than 1:160.
   - Hepatitis B surface antigen, hepatitis C antibody, HIV antibody, and Treponema pallidum antigen test were all negative.
   - Alanine aminotransferase is well within the normal ranges.

Guideline of clinical application

1. Indication: Severe and critically COVID-19 patients, and patients suffering advanced stages of the disease.
   - Duration of the disease is within three weeks, novel coronavirus virus nucleic acid test is positive with viremia.
   - Severely and critically ill COVID-19 patients assessed by clinicians.
   - Patients with long-term (more than 4 weeks) positive of novel coronavirus nucleic acid test (for details please refer to patient 2 in Figure 2).
2. Contraindication
   - Congenital IgA deficiency.
   - A history of allergy including plasma infusion, human plasma protein products, sodium citrate. Plasma inactivated by methylene blue virus is strictly prohibited in patients with methylene blue allergy. Other history of severe allergies and contraindications.
   - At the end of critical illness with irreversible multiple organ failure. Other conditions that are not suitable for infusion assessed by clinicians.
3. Dosage of infusion: According to the clinical status and the patient's weight. Usually the infusion dose is 200-500ml (4-5ml/kg).
4. Principle of infusion
   - According to the principle of cross-matching of blood, ABO homogenous plasma is preferred.
   - A slow infusion is required at the first 15 minutes in order to pay attention to the occurrence of adverse reactions of blood transfusion.
5. Risk disclosure and sign the informed consent

The retrospective cohort study of the convalescent plasma transfusion

The individual CP therapeutic process and outcomes of the 19 patients treated with CP transfusion were shown in Figure 2A. It can be clearly seen that symptoms in the 19 patients were all improved according to the different shades of color, which signifies the severity of the disease. Four critically ill patients with negative detection of viral nucleic acid (ID: 2, 6, 7, 17) also showed a good response to the CP treatment. By analyzing the titer of neutralizing antibody from the donors and the therapy response of the COVID-19 patients, we found that the plasma from the donors with a higher neutralizing antibody titer had a better treatment response ($p=0.0017$) (Figure 2B). Consistent with previous studies, CP treatment could improve the clinical outcomes through neutralizing viremia and decrease the viral load. According to the latest treatment guidelines, two consecutive negative viral nucleic acid tests can be regarded as the standard of discharge. Our results showed that the viral nucleic acid tests turned negative immediately after the CP treatment in the critically ill patients (ID: 4, 5, 8, 9, 10, 12, 18) and the moderately/severely ill patients with persistently positive detection of viral nucleic acid for more than three weeks (ID: 3, 13, 14, 15, 16, 19) (Figure 2A). All the 19 patients treated with CP transfusion in our study were survived, and showed a significantly lower case-fatality rate compared to the control group (0% vs. 19%, $p=0.031$). The survival curves of the exposure group and control group were shown in Figure 2C.

DISCUSSION

The use of convalescent plasma has a long history. At the end of the 19th century, researchers found that recovery patients' plasma was effective in diphtheria and tetanus patients. Use of convalescent plasma has been studied in outbreaks of other respiratory infections, including the 2003 SARS-CoV-1, 2009-2010 H1N1 influenza virus pandemic and the 2012 MERS-CoV epidemic [6]. Previous studies showed a shorter hospital stay and lower mortality with no adverse events or complications in patients who treated with convalescent plasma treatment than those who were not [7]. Additionally, viral load after convalescent plasma treatment was significantly lower on

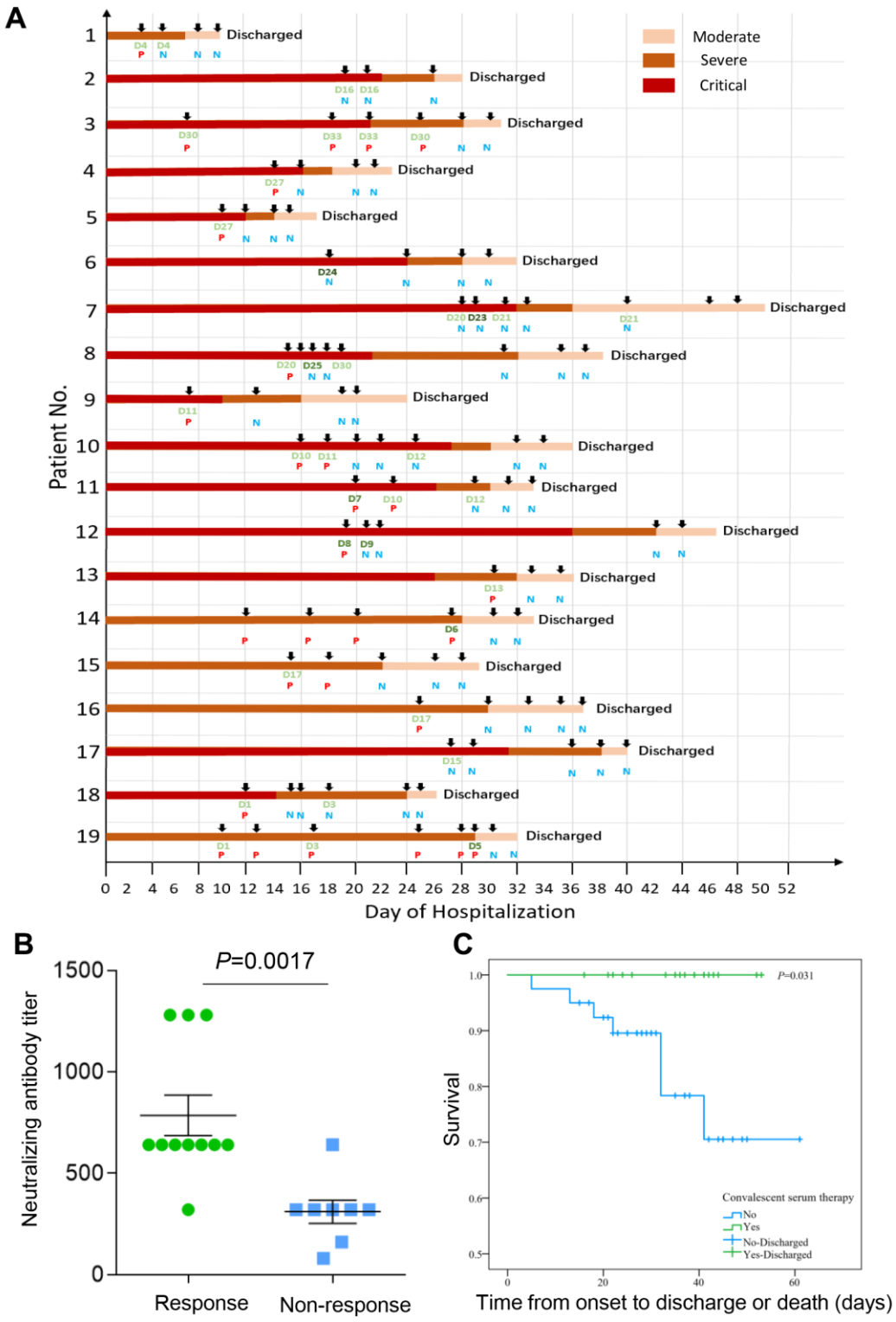

Figure 2. (**A**) Outcomes for individual patients included in 19 cases. Donor and receiver detail information see Supplementary Table 1; P, Nucleic acid test positive; N, Nucleic acid test negative; D, Donor patient (200ml); D, Donor patient (400ml). (**B**) The relationship between titer of neutralizing antibody from the donors and the therapy response of the COVID-19 patients. The plasma from the donors with a higher neutralizing antibody titer had a better treatment response (p=0.0017). The clinical symptoms were significantly improved and viral nucleic acid tests turned negative within five days after CP treatment was defined as "Response", otherwise it was "Non-response". (**C**) The survival curves of the exposure group and control group. All the 19 patients treated with CP transfusion in our study survived, and showed a significantly lower case-fatality rate compared to the control group (0% vs. 19%, *P*=0.031).

days 3, 5, and 7 after intensive care unit admission [8]. What's more, we found that patients with long-term positive nucleic acid test of novel coronavirus turn negative earlier after convalescent plasma treatment than those who without convalescent plasma treatment. Furthermore, asymptomatic patients with hypoimmunity, such as the elderly, children and patients with underlying diseases such as diabetes, hepatitis, AIDS, heart disease, tuberculosis, malignant tumor, etc., preferred to use convalescent plasma once the nucleic acid test was positive.

In our study, 19 COVID-19 patients were treated with CP and recovered quickly from the disease. The clinical symptoms were significantly improved as the individual critical/severe illness condition turned to moderate after CP treatment. The viral shedding was minimized and the clinical conditions of these patients improved as indicated by the viral nucleic acid test. We also found that the CP treatment was more effective in the patients with a higher neutralizing antibody titer transfusion. More importantly, all the patients in exposure group were survived and discharged, suggesting that the CP treatment was associated with a better outcome and a lower fatality.

In the war of fighting against emerging and pandemic COVID-19, the advantages of the convalescent plasma have been validated by the practice and clinical outcome in China. Therefore, it provided an unprecedented opportunity to perform clinical studies and trials of the efficacy of convalescent plasma transfusion during the pandemic of COVID-19. Meanwhile, the convalescent plasma transfusion is worthy of application and promotion in SARS-CoV-2-infected patients globally due to its safety and efficacy.

MATERIALS AND METHODS

A retrospective cohort study was conducted from January 22, 2020 to February 28, 2020. 19 patients with COVID-19 in Hunan Province confirmed by real-time viral RNA test and clinical manifestation were hospitalized and received transfusions from the CP donors. To compare the effectiveness of treatment defined by fatality rate, a control group comprised of 43 COVID-19 patients who did not receive CP treatment was selected from Hunan (23 cases) and Hubei (20 cases) provinces using a stratified random sampling method by age, gender and severity of the disease. Baseline characteristics (age, gender, severity, admission date, time from onset to hospitalization, length of days among survivors and time from hospitalization to death) of patients between the exposure group and control group were shown in Supplementary Tables 2, 3. The study was approved by the ethics committees of Xiangya Hospital, Central South University (approval #202002024) and other local hospitals. Written informed consent was obtained from each patient.

AUTHOR CONTRIBUTIONS

Xi Yuan, Zhimin Zhang collected the data. Shiyao Pei and Mingzhu Yin drafted the manuscript. Run Yao, Yubin Xie, Minxue Shen contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content and approved the final version of the manuscript. Bijuan Li, Xiang Chen were involved in data cleaning, and verification. All authors have read and approved the final manuscript.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

REFERENCES

1. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395:507–13. https://doi.org/10.1016/S0140-6736(20)30211-7 PMID:32007143

2. WHO. Coronavirus disease (COVID-2019) situation reports. 2020.

3. Chan KW, Wong VT, Tang SC. COVID-19: an update on the epidemiological, clinical, preventive and therapeutic evidence and guidelines of integrative Chinese-western medicine for the management of 2019 novel coronavirus disease. Am J Chin Med. 2020; 48:737–62. https://doi.org/10.1142/S0192415X20500378 PMID:32164424

4. Clinical treatment of COVID-19 Convalescent Plasma (the second trial edition). http://www.nhc.gov.cn/yzygj/s7658/202003/61d608a7e8bf49fca418a6074c2bf5a2.shtml.

5. FDA. Investigational covid-19 convalescent plasma—emergency INDs. https://www.fda.gov/vaccines-blood-biologics/investigational-new-drug-ind-or-device-exemption-ideprocess-cber/investigational-covid-19-convalescent-plasma-emergency-inds.

6. Roback JD, Guarner J. Convalescent plasma to treat COVID-19: possibilities and challenges. JAMA. 2020; 323:1561–62. https://doi.org/10.1001/jama.2020.4940 PMID:32219429

7. Soo YO, Cheng Y, Wong R, Hui DS, Lee CK, Tsang KK, Ng MH, Chan P, Cheng G, Sung JJ. Retrospective comparison of convalescent plasma with continuing high-dose methylprednisolone treatment in SARS patients. Clin Microbiol Infect. 2004; 10:676–78. https://doi.org/10.1111/j.1469-0691.2004.00956.x PMID:15214887

8. Hung IF, To KK, Lee CK, Lee KL, Chan K, Yan WW, Liu R, Watt CL, Chan WM, Lai KY, Koo CK, Buckley T, Chow FL, et al. Convalescent plasma treatment reduced mortality in patients with severe pandemic influenza A (H1N1) 2009 virus infection. Clin Infect Dis. 2011; 52:447–56. https://doi.org/10.1093/cid/ciq106 PMID:21248066

SUPPLEMENTARY MATERIALS

Supplementary Tables

Supplementary Table 1. Detection of novel coronavirus antibody: Colloidal gold label technology and ELISA.

Sample No.	Colloidal gold label technology		ELISA	
	IgG	IgM	IgG	IgM
1	+	-	640	1280
2	+	-	80	640
3	+	-	210	1280
4	±	-	80	320
5	+	-	160	2560
6	+	-	160	2560
7	±	-	80	-
8	+	-	80	2560
9	-	-	40	320
10	+	-	320	-
11	+	-	80	-
12	±	-	40	-
13	+	-	160	650
14	+	-	160	2560
15	+	-	160	1280
16	±	-	40	-
17	+	±	640	2560
Positive rate	94.1%	5.9%	100%	70.6%

Coincidence Rate: IgG 94.12%.
IgM 29.41%.

Dilution Ratio: Colloidal gold 1:8.
ELISA 1:10.

Supplementary Table 2. Characteristics of the patients of exposure group and control group.

Characteristics	Exposure group	Control group		*P*
	Hunan (N=19)	Hunan (N=23)	Hubei (N=20)	
Age (age), mean±SD	66.3±15.3	57.3±15.0	69.1±14.3	0.030
Sex, n (%)				
Male	11 (57.9)	13 (56.5)	12 (60.0)	0.964
Female	8 (42.1)	10 (35.1)	8 (40.0)	
Severity, n (%)				
Mild-to-moderate	0 (0.0)	0 (0.0)	0 (0.0)	0.053
Severe	6 (31.6)	1 (4.3)	6 (30.0)	
Critical	13 (68.4)	22 (95.7)	14 (70.0)	
Admission date				
First case	22 Jan 2020	20 Jan 2020	3 Feb 2020	
Last case	28 Feb 2020	6 Feb 2020	24 Feb 2020	
Time from onset to hospitalization (days), median (IQR)	4.5 (3.0–7.7)	5.0 (3.0–8.0)	13.0 (8.5–15.0)	<0.001
Length of stay (days) among survivors, median (IQR)	32.5 (24.5–37.7)	20.0 (17.0–21.0)	29.0 (26.0–31.3)	<0.001
Time from hospitalization to death (days), median (IQR)	N/A	10.0 (3.3–22.7)	22.0 (19.0–22.0)	0.157

Supplementary Table 3. The demographic characteristics of the donors.

Donors no.	Blood center	Gender	Age	Donated plasma volume, ml	Blood type	IgM/IgG	Neutralizing antibody titer
D1	Department of Blood Transfusion Laboratory of Changsha Blood Center	Male	40	400	A	Negative/ Positive	80
D3	Department of Blood Transfusion Laboratory of Changsha Blood Center	Male	41	400	A	Positive/ Positive	320
D4	Department of Blood Transfusion Laboratory of Changsha Blood Center	Male	30	400	B	Negative/ Positive	1280
D5	Department of Blood Transfusion Laboratory of Changsha Blood Center	Male	29	400	A	Positive/ Positive	640
D6	Department of Blood Transfusion Laboratory of Changsha Blood Center	Male	38	400	A	Positive/ Positive	1280
D7	Department of Blood Transfusion Laboratory of Zhuzhou Blood Center	Male	47	400	A	Positive/ Positive	320
D8	Department of Blood Transfusion Laboratory of Zhuzhou Blood Center	Male	30	400	A	Positive/ Positive	640
D9	Department of Blood Transfusion Laboratory of Zhuzhou Blood Center	Female	26	400	O	Positive/ Positive	160
D10	Department of Blood Transfusion Laboratory of Zhuzhou Blood Center	Female	41	400	A	Did not detected	Did not detected
D11	Department of Blood Transfusion Laboratory of	Female	44	400	A	Negative/ Positive	640

	Zhuzhou Blood Center						
D12	Department of Blood Transfusion Laboratory of Zhuzhou Blood Center	Female	23	400	AB	Negative/ Positive	640
D13	Department of Blood Transfusion Laboratory of Zhuzhou Blood Center	Male	31	400	AB	Positive/ Positive	320
D15	Department of Blood Transfusion Laboratory of Yueyang Blood Center	Female	49	300	B	Positive/ Positive	640
D16	Department of Blood Transfusion Laboratory of Yueyang Blood Center	Male	43	400	O	Positive/ Positive	640
D17	Department of Blood Transfusion Laboratory of Yueyang Blood Center	Male	22	400	O	Positive/ Positive	640
D20	Department of Blood Transfusion Laboratory of Loudi Blood Center	Female	36	400	O	Positive/ Positive	1280
D21	Department of Blood Transfusion Laboratory of Loudi Blood Center	Female	38	400	A	Weekly Positive/ Positive	320
D23	Department of Blood Transfusion Laboratory of Loudi Blood Center	Male	41	400	O	Weekly Positive/ Weekly Positive	80
D24	Department of Blood Transfusion Laboratory of Loudi Blood Center	Male	42	400	A	Positive/ Positive	320
D25	Department of Blood Transfusion Laboratory of Loudi Blood Center	Male	40	400	B	Weekly Positive/ Positive	320
D27	Department of Blood Transfusion Laboratory of Xiangtan Blood Center	Male	47	400	O	Positive/ Positive	640
D30	Department of Blood Transfusion Laboratory of Shaoyang Blood Center	Male	24	300	A	Did not detected	Did not detected
D33	Department of Blood Transfusion Laboratory of Shaoyang Blood Center	Female	22	200	A	Negative/ Positive	320

Research Paper

The effectiveness of continuous renal replacement therapy in critical COVID-19 patients with cytokine release syndrome: a retrospective, multicenter, descriptive study from Wuhan, China

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ABSTRACT

Background: Coronavirus disease (COVID-19) has spread rapidly since 2019. Approximately 15% of the patients will develop severe complications such as multiple organ disease syndrome related to cytokine release syndrome (CRS). Continuous renal replacement therapy (CRRT) can remove inflammatory cytokines through filtration or adsorption. We evaluated the effectiveness of CRRT in COVID-19 patients with CRS.
Methods: This retrospective, multicenter, descriptive study included 83 patients with CRS from three hospitals in Wuhan.
Results: In COVID-19 patients with CRS, the fatality rate was even higher in CRRT group (P=0.005). However, inflammatory markers such as C-reactive protein, neutrophil counts, and D-dimer decreased after CRRT (P<0.05). Results of Lasso model showed that tracheotomy (β -1.31) and convalescent plasma (β -1.41) were the protective factors. In contrast, CRRT (β 1.07), respiratory failure (β 1.61), consolidation on lung CT (β 0.48), acute kidney injury (AKI) (β 0.47), and elevated neutrophil count (β 0.02) were the risk factors for death.
Conclusions: Our results showed that although CRRT significantly reduced the inflammation, it did not decrease the fatality rate of patients with CRS. Therefore, the choice of CRRT indication, dialysis time and dialysis mode should be more careful and accurate in COVID-19 patients with CRS.

INTRODUCTION

An outbreak of coronavirus disease (COVID-19) in December 2019 in Wuhan, China, has developed into a global pandemic. COVID-19 is caused by infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus [1]. Most patients with COVID-19 present with respiratory symptoms, including dyspnea and fever, and approximately 15% of patients develop severe complications including injury to the liver, heart, and kidneys [2, 3]. Although the treatment of COVID-19 has been closely investigated, no uniform clinical treatment protocol is available yet. Therefore, it is imperative that an effective treatment is found as soon as possible [4].

Cytokine release syndrome (CRS) plays a vital role in the occurrence and disease progression of the COVID-19 [5]. It has been confirmed that there is a substantial increase in inflammatory cytokines in COVID-19 patients in a critical condition, and this increase is related to the SARS-CoV-2 nucleic acids [6]. Acute respiratory distress syndrome (ARDS) is the main cause

of death in COVID-19, associated with a massive inflammatory response mediated by the CRS [7]. SARS and Middle East respiratory syndrome (MERS) have also been shown to be associated with CRS and are closely linked to mortality [8]. CRS is a systemic inflammatory response that occurs mainly in cases of severe infection and is manifested by a sudden increase in the production of a large number of inflammatory factors, such as interleukin-2 (IL-2), IL-4, IL-6, and monocyte chemoattractant protein-1 [9]. Among these inflammatory cytokines, IL-1 and IL-6 are the main pathogenic factors. The cutoff value of specific inflammatory cytokines for CRS in patients with COVID-19 is yet to be defined. Recent research shows that an IL-6 value greater than 100 pg/mL carries the risk of death, and artificial-liver blood-purification therapy can be considered when IL-6 is five times the normal limit [6, 10].

Although there are no standard diagnostic criteria for CRS, some treatments based on managing CRS have been widely applied clinically. These include antagonism at the IL-6 receptor (using the antagonist Tocilizumab), artificial-liver blood-purification therapy that can remove cytokines from the circulation, and continuous renal replacement therapy (CRRT) can remove inflammatory factors such as cytokines from the circulation via filtration and adsorption [11]. Tocilizumab has been proved to be effective in COVID-19 at the initial stages [12, 13]. CRRT has been used in the treatment of COVID-19 patients with multiple organ failure, especially when accompanied by refractory CRS, and in some critical patients with acute kidney injury (AKI). One case report suggests that CRRT is initially effective in the treatment of patients with COVID-19 [14]. However, as little data on this topic exists, there is no definitive conclusion regarding the effectiveness of CRRT. In this study, we retrospectively collected and analyzed the clinical data of COVID-19 patients with CRS from three hospitals in Wuhan, to determine the effectiveness of CRRT.

RESULTS

Of the 83 cases included in our study, 67 were classified as critical, and 16 as non-critical. Of the 67 critical patients, 38 cases were treated with CRRT during hospitalization. A total of 45 patients died, and 36 were cured or improved. The outcome of two patients is unknown.

Patients demographics

The mean age of the patients was 67.3±12.6 years. Almost three-quarters (73.5%) of the patients were male; 67 were critical cases, and 13.3% of the patients had a history of smoking. The underlying medical conditions of the patients included hypertension (55.4%), diabetes (24.1%), tumors (6%), and chronic obstructive pulmonary disease (1.2%). Pulmonary CT revealed that 51.7% of the critical patients had consolidation, and 73.3 % of non-critical patients had ground-glass changes in the lungs. The mean peak creatinine value during hospitalization was 133.2 pg/mL (range, 72.8 to 276.7). AKI was present in 60.2%, MODS in 36.1%, and ARDS in 42.2% of the patients. Compared to the non-critical groups, the critical group had more patients with an IL-6 value >4000 pg/mL (29.9% vs.18.8%) (Table 1).

Patients in the critical group had a significantly higher WBC count (P=0.001), neutrophil count (P<0.001), neutrophil percentage (P<0.001), LDH (P<0.001), peak creatinine (P=0.01), high-sensitivity troponin I (P<0.001), CRP (P<0.001), ferritin (P=0.001) and procalcitonin (PCT) (P=0.043) than those in the non-critical group. Patients in the critical group had significantly lower lymphocyte counts (P=0.018) and albumin levels (P=0.015) than patients in the non-critical group (Table 1).

Treatments such as CRRT (P=0.001), antibiotics (P=0.002), hormones (P=0.004), globulin therapy (P<0.001), invasive mechanical ventilation (P<0.001) and non-invasive ventilation (P<0.001), were each more commonly used in critical patients than in non-critical patients. Moreover, critical patients had a higher incidence of AKI (P<0.001), respiratory failure (P<0.001), ARDS (P<0.001), MODS (P<0.001), gastrointestinal bleeding (P=0.004), acute liver dysfunction (P<0.001) and acute myocardial injury (P<0.001) (Table 1).

The role of CRRT in critical COVID-19 patients with CRS

Over half of the critical patients (38/67) were treated with CRRT. Those who received CRRT had a lower blood platelets (PLT) (P=0.023), and lower albumin (P=0.041), a higher peak creatinine (P=0.018), and a greater incidence of MODS (P<0.001) than those who did not receive CRRT. More patients receiving CRRT also underwent tracheal cannulation (P=0.003) or invasive mechanical ventilation (P=0.002). Most patients who received CRRT (64.7%) had pulmonary consolidations on CT (Table 2). Unexpectedly, mortality was higher in the CRRT group than that in the non-CRRT group (P=0.005).

Compared to the non-CRRT group, the CRRT group had more patients with an IL-6 value >4000 pg/mL (24.1% vs. 34.2%). The blood oxygen saturation in patients who received CRRT was lower than in the non-CRRT group

Table 1. The general characteristics of 83 COVID-19 patients by disease severity.

Variables		Total(n=83)	Critical (n=67)	Non-critical (n=16)	$t/\chi^2/Z$	*P*
Age (years)		67.3±12.6	67.6±12.3	65.9±13.9	-0.490[a]	0.625
Sex						
	Female	22(26.5)	16(23.9)	6(37.5)	0.630[c1]	0.427
	Male	61(73.5)	51(76.1)	10(62.5)		
Smoking		11(13.3)	8(15.7)	3(21.4)	0.011[c1]	0.916
Clinical outcomes						
	Cure/Improved	36(43.4)	22(33.3)	14(93.3)	17.820[c2]	<0.001
	Death	45(54.2)	44(66.7)	1(6.7)		
Underlying conditions						
	Diabetes	20 (24.1)	14(20.9)	6(37.5)	1.145[c1]	0.285
	Hypertension	46(55.4)	37(55.2)	9(56.2)	0.006[c2]	0.941
	Tumor	5(6.0)	5(7.5)	0(0.0)	-	0.578[f]
	COPD	1(1.2)	1(1.5)	0(0.0)	-	1.000[f]
Hospitalization time (days)		20(11,35)	19(11,30.75)*	34(22,47.5)*	683.0[b]	0.022
Laboratory test results						
	SO2 (%)	93(87,95)	92.5(85,95)*	93(88,95)*	413.5[b]	0.591
	PLT (10^9/L)	158(107,216.5)	153(105.5,212.5)	180(135,233.25)	622.0[b]	0.324
	WBC count (10^9/L)	8.21(6.32,11.84)	8.98(6.8,12.88)	5.58(3.68,7.08)	254.0[b]	0.001
	Neutrophil count (10^9/L)	7.65(4.67,11.17)	8.01(5.48,12.01)	3.47(2.25,5.84)	217.0[b]	<0.001
	Neutrophil %	87.9(75,91.4)	89.9(80.1,92.1)*	68.85(63.42,77.65)*	94.5	<0.001
	Lymphocyte count (10^9/L)	0.64(0.460.98)	0.59(0.42,0.96)	0.93(0.71,1.08)	741.5[b]	0.018
	Albumin (g/L)	30.6(26.2,33.95)	29.4(25.8,33.85)	33.75(30.68,36.1)	746.5[b]	0.015
	LDH (U/L)	375(263,534.5)	434(328.5,592)	248.5(217.5,266.25)	122.0[b]	<0.001
	Maximum creatinine value during hospitalization (μmol/L)	133.2(72.75,276.7)	146(92.45,291.35)	73.4(68.62,82.3)	312.0[b]	0.010
	high-sensitivity troponin I	26(9.5,111.75)	36(12.6,140.5)*	4.45(1.65,9.8)*	92.0[b]	<0.001
	D-dimer (ug/mL)	3.14(0.94,7.39)	3.31(0.88,8)*	2.67(1.66,4.97)	484.0[b]	0.672
	CRP (mg/L)	85.35(33.41,134.12)	96.7(43.8,143.8)*	14(2.58,49.5)*	202.0[b]	<0.001
	Ferritins (μg/L)	1271.82(726.64,2000)	1614(837,2000)*	565.1(303.82,957.31)*	80.0[b]	0.001
	PCT (ng/mL)	0.23(0.11,0.41)	0.24(0.12,0.41)*	0.06(0.05,0.26)*	206.0[b]	0.043
	Maximum IL-6 (pg/mL)					
	$<P_{25}$ (<=250)	21(25.3)	15(22.4)	6(37.5)	-	0.426[c3]
	P_{25}~P_{75} (250 -4000)	39(47.0)	32(47.8)	7(43.8)		
	$>=P_{75}$(>=4000)	23(27.7)	20(29.9)	3(18.8)		
Lung CT main performance	Ground-glass	40(48.2)	29(48.3)	11(73.3)	3.013[c2]	0.083
	Consolidation	35(42.2)	31(51.7)	4(26.7)		
Treatments						
	CRRT	40(48.2)	38(56.7)	2(12.5)	10.144[c2]	0.001
	Antiviral	80(96.4)	64(98.5)	16(100.0)		1.000[c3]
	Tracheal cannula	56(67.5)	56(88.9)	0(0.0)	41.258[c1]	<0.001
	Tracheotomy	13(15.7)	13(21.3)	0(0.0)	2.275[c1]	0.131
	Antibiotic	73(88.0)	62(96.9)	11(68.8)	9.403[c1]	0.002
	Hormone	53(63.9)	47(78.3)	6(37.5)	8.139[c1]	0.004
	Globulin	41(49.4)	38(67.9)	3(18.8)	12.240[c1]	<0.001
	ACEI/ARB	6(7.2)	4(7.7)	2(14.3)		0.600[f]
	Convalescent plasma	10(12.0)	10(16.7)	0(0.0)	1.623[c1]	0.203
	Traditional Chinese medicine and pharmacy	27(32.5)	18(32.7)	9(69.2)	5.852[c2]	0.016
	High flow nasal catheter oxygen inhalation	63(75.9)	49(80.3)	14(87.5)	0.089[c1]	0.766
	Non-invasive ventilation	40(48.2)	40(65.6)	0(0.0)	21.834[c2]	<0.001
	Invasive mechanical ventilation	60(72.3)	59(89.4)	1(6.2)	41.212[c1]	<0.001
	ECMO	9(10.8)	9(14.3)	0(0.0)	1.358[c1]	0.244

Complications						
	AKI	50(60.2)	49(74.2)	1(6.2)	25.020[c2]	<0.001
	Respiratory failure	61(73.5)	57(91.9)	4(26.7)	27.417[c1]	<0.001
	Gastrointestinal bleeding	26(31.3)	25(45.5)	1(6.2)	8.208[c2]	0.004
	Acute liver dysfunction	45(54.2)	41(82.0)	4(25.0)	18.153[c2]	<0.001
	Acute myocardial injury	58(69.9)	56(88.9)	2(12.5)	34.337[c1]	<0.001
	ARDS	35(42.2)	35(62.5)	0(0.0)	19.459[c1]	<0.001
	MODS	30(36.1)	30(62.5)	0(0.0)	16.953[c1]	<0.001

Note: [a]: t value by t test; [b]: Z value by Wilcoxon rank sum test; [c1]: continuity corrected χ^2 value by χ^2 test; [c2]: χ^2value by χ^2 test; [f]: Fisher exact probability test p value.

*: contains missing values.

PLT: Blood platelets; WBC count: White blood cell count; SO2: Blood oxygen saturation; LDH: Lactate dehydrogenase; CRP: C-reactive protein; PCT: Procalcitonin; IL6: Interleukin 6; COPD: Chronic obstructive pulmonary disease; AKI: acute kidney injury.

Table 2. The general characteristics of 67 critical COVID-19 patients by CRRT.

Variable		Total	CRRT (n=38)	Non-CRRT (n=29)	$t/\chi^2/Z$	*P*
Age (years)		67.6±12.3	66.9±11.8	68.6±13.0	0.552[a]	0.583
Sex						
	Female	16	7(18.4)	9(31.0)	1.440[c2]	0.230
	Male	51	31(81.6)	20(69.0)		
Smoking		8	6(21.4)	2(8.7)		0.269[f]
Clinical outcomes						
	Cure/Improve	22	7(18.9)	15(51.7)	7.873[c2]	0.005
	Death	44	30(81.1)	14(48.3)		
Underlying conditions						
	Diabetes	14	7(18.4)	7(24.1)	0.325[c2]	0.568
	Hypertension	37	19(50.0)	18(62.1)	0.969[c2]	0.325
	Tumor	5	2(5.3)	3(10.3)		0.645[f]
	COPD	1	0(0.0)	1(3.4)		0.433[f]
Hospitalization time (days)		23.3±16.6	23.6±15.6*	23.0±18.1	477.5[b]	0.449
Laboratory test results						
	SO2 (%)	92.5(85,95)	90(84.5,95)*	93(88,95.5)*	462.5[b]	0.343
	PLT (10^9/L)	153(105.5,212.5)	127(98.25,179)	185(112,247)	731.0[b]	0.023
	WBC count (10^9/L)	9.9±4.4	9.8±3.9	10.0±4.9	0.180[a]	0.858
	Neutrophil count (10^9/L)	8.80±4.30	8.80±3.89	8.80±4.87	-0.003[a]	0.997
	Neutrophil %	89.9(80.1,92.1)	89.95(86.6,93.13)*	89.8(78.5,91.4)	420.5[b]	0.321
	Lymphocyte count (10^9/L)	0.59(0.42,0.96)	0.55(0.39,0.72)	0.64(0.48,1.12)	666.5[b]	0.146
	Albumin (g/L)	29.88±5.51	28.68±4.79	31.44±6.06	2.080[a]	0.041
	LDH (U/L)	434(328.5,592)	472(339.25,661)	430(321,502)	451.0[b]	0.208
	Maximum creatinine value during hospitalization (μmol/L)	146(92.45,291.35)	198.85(123.62,340.95)	132.7(63.1,214)	364.0[b]	0.018
	High-sensitivity troponin I	36(14.6,140.5)	46.6(19.9,184.25)*	23.7(12.55,70.82)*	354.0[b]	0.229
	D-dimer (ug/mL)	3.31(0.88,8)	2.26(0.92,8)*	3.53(0.88,6.98)	513.5[b]	0.915
	CRP (mg/L)	96.7(43.8,143.8)	97.76(54.34,143.57)*	89.16(33.93,143.8)	480.0[b]	0.583
	Ferritins (μg/L)	1614(837,20000)	1618.59(907.86,2000)*	1447.99(851.62,2000)*	183.0[b]	0.522
	PCT (ng/mL)	0.24(0.12,0.41)	0.3(0.14,0.49)*	0.23(0.12,0.27)*	276.5[b]	0.120
	Maximum IL-6 (pg/mL)					
	<P_{25} (<=250)	15	11(28.9)	4(13.8)	4.438[c2]	0.109
	P_{25}~P_{75} (250 -4000)	32	14(36.8)	18(62.1)		
	>=P_{75}(>=4000)	20	13(34.2)	7(24.1)		
Lung CT main performance	Ground glass	29	12(35.3)	17(65.4)	5.342[c2]	0.021
	Consolidation	31	22(64.7)	9(34.6)		

Treatments						
	Antiviral	64	35(97.2)	29(100.0)		1.000[f]
	Tracheal cannula	56	34(100.0)	22(75.9)		0.003[f]
	Tracheotomy	13	8(24.2)	5(17.9)	0.368[c2]	0.544
	Antibiotic	62	35(97.2)	27(96.4)		1.000[f]
	Hormone	47	30(83.3)	17(70.8)	1.326[c2]	0.250
	Globulin	38	24(77.4)	14(56.0)	2.911[c2]	0.088
	ACEI/ARB	4	1(3.3)	3(13.6)		0.299[f]
	Convalescent plasma	10	7(21.2)	3(11.1)	0.485[c1]	0.486
	Traditional Chinese medicine and pharmacy	18	8(25.8)	10(41.7)	1.546[c2]	0.214
	High flow nasal catheter oxygen inhalation	49	25(75.8)	24(85.7)	0.950[c2]	0.330
	Non-invasive ventilation	40	23(65.7)	17(65.4)	0.001[c2]	0.979
	Invasive mechanical ventilation	59	37(100.0)	22(75.9)		0.002[f]
	ECMO	9	6(17.1)	3(10.7)	0.131[c1]	0.717
Complications						
	AKI	49	30(81.1)	19(65.5)	2.059[c2]	0.151
	Respiratory failure	57	35(94.6)	22(88.0)		0.385[f]
	Gastrointestinal bleeding	25	16(48.5)	9(40.9)	0.306[c2]	0.580
	Acute liver dysfunction	41	26(81.2)	15(83.3)	0.000[c1]	1.000
	Acute myocardial injury	56	32(88.9)	24(88.9)		1.000[f]
	ARDS	35	25(71.4)	10(47.6)	3.175[c2]	0.075
	MODS	30	26(81.2)	4(25.0)	14.400[c2]	<0.001

Note: [a]: t value by t test; [b]: Z value by Wilcoxon rank sum test; [c1]: continuity corrected χ^2 value by χ^2 test; [c2]: χ^2value by χ^2 test; [f]: Fisher exact probability test p value.

*: contains missing values.

PLT: Blood platelets; WBC count: White blood cell count; SO2: Blood oxygen saturation; LDH: Lactate dehydrogenase; CRP: C-reactive protein; PCT: Procalcitonin; IL6: Interleukin 6; COPD: Chronic obstructive pulmonary disease; AKI: acute kidney injury.

[90 (84.5,95)% vs.93 (88,95.5)%] and the lymphocyte count [0.55 (0.39,0.72) 10^9g/L vs. 0.64 (0.48,1.12) 10^9g/L] followed the same pattern, demonstrating lower values in the CRRT patients. The CRP was higher in the CRRT than in the non-CRRT group [97.7 (54.34,143.57) mg/L vs. 89.16 (33.93,143.8) mg/L)]. Moreover, the incidence of AKI and ARDS in critical patients treated with CRRT was 81.1% and 71.4%, respectively, which was higher than the corresponding figures of non-CRRT (Table 2). Compared with the non-CRRT patients, the patients in the CRRT group appeared to be in a worse condition, although there was no statistical difference in the indicators mentioned above.

For the 38 patients treated with CRRT, the changes of inflammation-related indicators before and after CRRT were compared. These included the count and percentage of neutrophils, WBC counts, lymphocytes counts, and levels of IL-6, CRP, D-dimer, and PCT. The analysis showed that after CRRT, WBC counts (P=0.039), neutrophil counts (P=0.014), CRP (P=0.049), D-dimer (P=0.006) all declined significantly from the values before CRRT. However, lymphocytes, PCT and IL-6 did not change significantly (Table 3).

Factors related to mortality

The results from Lasso analysis indicated that patients with CRRT (β 1.07), consolidation of the lungs (β 0.48), respiratory failure (β 1.61), AKI (β 0.47), and elevated neutrophils (β 0.02) could have a higher the risk of death. Tracheotomy (β -1.31) and convalescent plasma (β -1.41) were found to be negatively correlated with death (Table 4).

Logistic regression analysis of 66 critical patients further showed that respiratory failure was an independent risk factor for death (odds ratio [OR], 0.06; 95% confidence interval [CI], 0.01-0.38, P<0.001), while tracheotomy was found to be an independent protective factor for death (OR, 68.72; 95% CI, 4.81-10404.57, P<0.001). Therefore, it is suggested that timely tracheotomy should be performed in critically ill patients with respiratory failure (Table 5).

DISCUSSION

In this study, we found that the fatality rate of critical COVID-19 patients with CRS who received CRRT was

Table 3. The effect of CRRT on inflammatory response.

Variable	Before	After	Difference	V	*P*	N
WBC count(10^9/L)	12.85(9.96,18.77)	8.42(4.44,15.06)	-4.43(-10.12,2.14)	176.5	0.039	34
Neutrophil counts	12.22(8.45,17.71)	7(3.47,12.19)	-4.24(-9.65,1.47)	134	0.014	32
Neutrophil %	92.2(88.88,94.80)	89(79.95,92.52)	-1.95(-5.05,0.62)	177	0.040	34
Lymphocyte counts (10^9/L)	0.54(0.36,0.96)	0.54(0.24,1.02)	-0.1(-0.28,0.20)	249	0.412	34
CRP (mg/L)	120.10(64.66,160)	63.6(42.11,128)	-37.1(-68.34,33.58)	126	0.049	31
PCT (ng/mL)	1.67(0.70,4.69)	2.58(0.69,6.50)	-0.86(-2.39,4.74)	246	0.747	32
D-dimer (ug/L)	7.08(2.29,12.14)	3.94(1.88,7.18)	-2.61(-6.04,0)	90	0.006	31
IL 6 (pg/L)	20.95(9.13,91.29)	30.34(16.08,416.02)	9.30(-13.75,287.23)	175	0.267	24

Note: V is the statistic of Wilcoxon signed rank test. N is the number of the patients.
WBC count: White blood cell count; CRP: C-reactive protein; PCT: Procalcitonin; IL6: Interleukin 6.

Table 4. The results of lasso regression model on the death outcome of the 66 critical patients with COVID-19.

Variables	β	OR*
CRRT	1.07	2.92
Tracheotomy	-1.31	0.27
Convalescent plasma	-1.41	0.24
Respiratory failure	1.61	4.99
Lung CT main performance	0.48	1.62
AKI	0.47	1.60
Neutrophil %	0.02	1.02

Note: AKI: acute kidney injury; WBC count: White blood cell count; LDH: Lactate dehydrogenase; CRP: C-reactive protein; *: OR value was calculated based on β, $OR=e^{\beta}$. Variables with positive β are risk factors of death outcome while those negative are protective.
In addition to the variables shown in the Table 4, these variables such as cough, dyspnea, hormone treatment, Invasive mechanical ventilation, acute myocardial injury, WBC count (10^9/L), Neutrophil count (10^9/L), Lymphocyte count (10^9/L), Albumin (g/L), LDH (U/L), maximum creatinine value during hospitalization(μmol/L), CRP (mg/L) and D-dimer (ug/mL) were also included into lasso model. However, their coefficients were shank to 0 by the model due to their negligible effects, therefore these variables were not included in the table.

Table 5. Results of the exact logistic regression on the death outcome of the 66 critical patients with COVID-19.

	coef	se(coef)	lower 0.95	upper 0.95	χ^2	*P*	OR (95%CI)
Tracheotomy(Yes)	- 2.73	0.99	- 4.80	- 0.97	9.45	<0.001	0.06 (0.01,0.38)
Respiratory failure(Yes)	4.23	1.87	1.57	9.25	10.79	<0.001	68.72 (4.81,10404.57)

higher than that of those who did not. This means that the clearance of inflammatory factors by CRRT did not improve the prognosis of CRS. Furthermore, the results from Lasso analysis also showed that CRRT was a dependent risk factor for death. The result may not have been expected, and is not consistent with some reports in the literature. For example, several case reports suggest that CRRT can improve the prognosis and may be a potential treatment for COVID-19 [15–17]. However, the results of Fominskiy E et al. also show that CRRT does not reduce the mortality of patients with COVID-19, and CRRT also carries a higher risk of in-hospital

death [18]. Therefore, whether CRRT can improve the prognosis of critical COVID-19 patients with CRS is still a controversial question.

In our study, the patients in the CRRT group were more seriously ill. Compared with the non-CRRT patients, they had a higher level of peak creatinine and had a higher incidence of MODS or ARDS. In addition, the unsuitable choice of dialysis timings or dialysis mode, and dialysis-related complications, such as bleeding caused by anticoagulation, might contribute to the high fatality rate in the CRRT group [19]. The therapeutic effect of CRRT is reduced when IL-6 suddenly increases to more than 100 pg/mL or when other complications are present [6, 20]. Therefore, when COVID-19 patients develop CRS, the use of CRRT should not be delayed. As currently recommended for interventional treatment of artificial-liver blood-purification, when IL-6 is five times the normal limit, the application of CRRT should be considered. If the characteristics of our cases and the current literature are considered together, IL-6 values greater than 100pg/ml could be an important critical index for intervention. However, the determination of the specific cutoff value of IL-6 for early intervention with CRRT requires further investigation [10]. Although CRRT did not improve the survival of critical COVID-19 patients with CRS, some inflammatory markers such as the CRP, D-dimer, WBC counts, and neutrophil counts decreased significantly after CRRT. It is suggested that CRRT can reduce inflammation in such patients.

The results from Lasso analysis indicated that AKI is a risk factor for death in critical COVID-19 patients with CRS. This result is consistent with the conclusion of a study conducted by Tongji Hospital, another hospital focused on the treatment of COVID-19 patients in Wuhan [21]. The incidence of AKI in our study was much higher than previously reported for ICU patients [2]. It is suggested that in addition to the lung, the kidney is also one of the most frequently affected organs in COVID-19 infections. Recent studies have confirmed that the expression of angiotensin-converting enzyme 2, a cell entry receptor of SARS-CoV-2, is very high in the kidney [22, 23]. In addition, the changes in hemodynamics and the damage done by inflammatory cytokines to the kidneys are also the reason for the high incidence of AKI in COVID-19 patients [24, 25]. So, the urinary system is a potential route for SARS-CoV-2 infection. In addition, the Lasso analysis suggests that consolidation on lung CT and elevated neutrophils also increase the risk of death.

The incidence of respiratory failure is higher in patients with critical COVID-19 (73.5%), and multiple regression analysis also confirms that respiratory failure is a risk factor for death. Tracheotomy was found to be protective for death among critical COVID-19 patients with CRS, indicating that patients with respiratory failure should undergo tracheotomy and invasive ventilation at the correct time.

In terms of treatment, in addition to CRRT and invasive ventilation, many other methods are used to treat these seriously ill patients. These treatments include convalescent plasma, hormones, globulin, and Chinese medicine, but the benefits are also controversial. In our study, convalescent plasma treatment might have been beneficial for critical COVID-19 patients with CRS, according to Lasso analysis. Hormones and globulin were commonly used treatments in severe SARS or MERS patients in the past. Over 78.3% of the critical patients in this study received hormone therapy for the treatment of COVID-19. Though the treatment with hormones was not found to be either a protective or a risk factor in this study, it should be carefully considered [26]. Regarding Chinese medicine, we did not see any protective factors in our results, which may be inconsistent with some reports [27]. The reason may be that most Chinese medicines are used in patients with mild or moderate symptoms, while most COVID-19 patients with CRS have severe or critical symptoms, and fewer of them are treated with Chinese medicine.

Our study has several limitations. First, though we have compiled cases from three hospitals that mainly treated patients with COVID-19, the sample size is still comparatively small. Second, although some confounding factors were compensated for, the influences of some unknown/unavailable factors cannot be completely excluded. Third, the medical records of two patients are incomplete, and the prognosis is unknown, resulting in missing data.

In conclusion, the fatality rate of CRRT patients did not decrease as expected, and even had an opposite trend in our study. The decision whether and how to use CRRT in COVID-19 patients with CRS should be carefully assessed. In the future, more studies and larger sample size are needed to evaluate the effect of CRRT on COVID-19 patients with CRS. Convalescent plasma therapy might be clinically considered in critical COVID-19 patients, and tracheotomy could be recommended for those who developed respiratory failure.

MATERIALS AND METHODS

Study design

In this retrospective, multi-center study, we analyzed results from 83 patients diagnosed with COVID-19 and CRS from December 2019 to July 2020, at three participating hospitals (Wuhan Union hospital, Wuhan

Jinyintan Hospital, and Wuhan First Hospital). The following inclusion criteria were applied: a laboratory diagnosis of COVID-19 (a positive throat swab nucleic acid test or positive serum COVID-19 specific antibody test), and a peak IL-6 value >100 pg/mL, or a peak IL-6 value of 50-100 pg/mL with concurrent ARDS or multiple organ disease syndrome (MODS). Clinical indications for CRRT include hyperkalemia, acidosis, multiple organ dysfunction, or severe CRS. This study was approved by the institutional ethics board of Wuhan First Hospital (W202003–2) and conducted in accordance with the Declaration of Helsinki. The requirement for informed consent was waived by the ethics board.

Definition

According to the New Coronavirus Pneumonia Prevention and Control Plan (seventh edition) published by the National Health Commission of China, COVID-19 can be classified as mild, moderate, severe, and critical. Mild infections are those that only have clinical manifestations but no abnormalities on computed tomography (CT) scan of the lungs. Patients with clinical and pulmonary CT manifestations are considered as moderate cases. Severe cases of COVID-19 are defined as those with a respiratory rate ≥ 30 breaths/min, blood oxygen saturation ≤ 93%, or arterial PO_2/oxygen concentration ≤ 300 mmHg. A patient with COVID-19 is considered to be critical when respiratory failure requires mechanical ventilation, or the patient experiences shock or multiple organ failure and is transferred to the intensive care unit. After careful evaluation, all the patients included in this study were classified as severe and critical cases and therefore were divided into critical and non-critical groups.

AKI was defined as an increase in serum creatinine by 26.52 mmol/L within 48 hours or by more than 50% from the baseline within 7 days [28].

Data collection

After careful review, data, including demographics, clinical characteristics, laboratory and radiological examinations and treatments, were extracted from the patients' medical records. Patients were classified as critical or non-critical, according to the criteria described in the definitions sections above. We retrieved the values of relevant CRRT indicators obtained before and after CRRT. All laboratory examinations of patients were carried out by trained physicians.

Statistical analyses

Categorical variables were presented as numbers and proportions, and the difference between the groups was determined using the chi-square test or Fisher's exact test. Continuous variables were presented as mean (SD) or median [interquartile (IQR)], and differences between the groups were determined using a two-sample t-test or Wilcoxon rank-sum test. Lasso and accurate logistic analysis were conducted to identify the factors related to death. The explanatory variables included treatments (CRRT, tracheotomy, convalescent plasma), respiratory failure, consolidation on lung CT, AKI, elevated neutrophil percentage, cough, dyspnea, hormone treatment, invasive mechanical ventilation, acute myocardial injury, White blood cell (WBC) count (x 10^9/L), neutrophil count (x 10^9/L), lymphocyte count (x 10^9/L), albumin (g/L), lactate dehydrogenase (LDH) (U/L), maximum creatinine value during hospitalization (μmol/L), C-reactive protein (CRP) (mg/L) and D-dimer (ug/mL) levels. Statistical significance was set as two-sided with $P < 0.05$. All the analyses were conducted using R software (version 3.6.2, R Foundation), and the Lasso and acute logistic analysis were performed with the glmnet and logistf package.

Data availability statement

The data underlying this article are available in the article and its online supplementary material.

AUTHOR CONTRIBUTIONS

JX designed the study and HX collected the data. HX and BS drafted the manuscript. JZ and YZ performed the statistical analysis. JX and JZ revised the manuscript. All the authors have reviewed and approved the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course

and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81.
https://doi.org/10.1016/S2213-2600(20)30079-5
PMID:32105632

2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506.
https://doi.org/10.1016/S0140-6736(20)30183-5
PMID:31986264

3. Cao X. COVID-19: immunopathology and its implications for therapy. Nat Rev Immunol. 2020; 20:269–70.
https://doi.org/10.1038/s41577-020-0308-3
PMID:32273594

4. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DS, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020; 382:1708–20.
https://doi.org/10.1056/NEJMoa2002032
PMID:32109013

5. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ, and HLH Across Speciality Collaboration, UK. COVID-19: consider cytokine storm syndromes and immunosuppression. Lancet. 2020; 395:1033–34.
https://doi.org/10.1016/S0140-6736(20)30628-0
PMID:32192578

6. Chen X. Detectable serum SARS-CoV-2 viral load (RNAaemia) is closely correlated with drastically elevated interleukin 6 (IL-6) level in critically ill COVID-19 patients. Clin Infect Dis. 2020; 71:1937–42.
https://doi.org/10.1093/cid/ciaa449
PMID:32301997

7. McGonagle D, Sharif K, O'Regan A, Bridgewood C. The role of cytokines including interleukin-6 in COVID-19 induced pneumonia and macrophage activation syndrome-like disease. Autoimmun Rev. 2020; 19:102537.
https://doi.org/10.1016/j.autrev.2020.102537
PMID:32251717

8. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Semin Immunopathol. 2017; 39:529–39.
https://doi.org/10.1007/s00281-017-0629-x
PMID:28466096

9. Shimabukuro-Vornhagen A, Gödel P, Subklewe M, Stemmler HJ, Schlößer HA, Schlaak M, Kochanek M, Böll B, von Bergwelt-Baildon MS. Cytokine release syndrome. J Immunother Cancer. 2018; 6:56.
https://doi.org/10.1186/s40425-018-0343-9
PMID:29907163

10. Zhang Y, Yu L, Tang L, Zhu M, Jin Y, Wang Z, Li L. A promising anti-cytokine-storm targeted therapy for COVID-19: the artificial-liver blood-purification system. Engineering (Beijing). 2020. [Epub ahead of print].
https://doi.org/10.1016/j.eng.2020.03.006
PMID:32292628

11. Ankawi G, Neri M, Zhang J, Breglia A, Ricci Z, Ronco C. Extracorporeal techniques for the treatment of critically ill patients with sepsis beyond conventional blood purification therapy: the promises and the pitfalls. Crit Care. 2018; 22:262.
https://doi.org/10.1186/s13054-018-2181-z
PMID:30360755

12. Giamarellos-Bourboulis EJ, Netea MG, Rovina N, Akinosoglou K, Antoniadou A, Antonakos N, Damoraki G, Gkavogianni T, Adami ME, Katsaounou P, Ntaganou M, Kyriakopoulou M, Dimopoulos G, et al. Complex immune dysregulation in COVID-19 patients with severe respiratory failure. Cell Host Microbe. 2020; 27:992–1000.e3.
https://doi.org/10.1016/j.chom.2020.04.009
PMID:32320677

13. Xu X, Han M, Li T, Sun W, Wang D, Fu B, Zhou Y, Zheng X, Yang Y, Li X, Zhang X, Pan A, Wei H. Effective treatment of severe COVID-19 patients with tocilizumab. Proc Natl Acad Sci USA. 2020; 117:10970–75.
https://doi.org/10.1073/pnas.2005615117
PMID:32350134

14. Ma J, Xia P, Zhou Y, Liu Z, Zhou X, Wang J, Li T, Yan X, Chen L, Zhang S, Qin Y, Li X. Potential effect of blood purification therapy in reducing cytokine storm as a late complication of critically ill COVID-19. Clin Immunol. 2020; 214:108408.
https://doi.org/10.1016/j.clim.2020.108408
PMID:32247038

15. Katagiri D, Ishikane M, Ogawa T, Kinoshita N, Katano H, Suzuki T, Fukaya T, Hinoshita F, Ohmagari N. Continuous renal replacement therapy for a patient with severe COVID-19. Blood Purif. 2021; 50:129–31.
https://doi.org/10.1159/000508062
PMID:32526746

16. Dastan F, Saffaei A, Mortazavi SM, Jamaati H, Adnani N, Samiee Roudi S, Kiani A, Abedini A, Hashemian SM. Continues renal replacement therapy (CRRT) with disposable hemoperfusion cartridge: A promising option for severe COVID-19. J Glob Antimicrob Resist. 2020; 21:340–41.
https://doi.org/10.1016/j.jgar.2020.04.024
PMID:32353526

17. Esmaeili Vardanjani A, Ronco C, Rafiei H, Golitaleb M, Pishvaei MH, Mohammadi M. Early hemoperfusion for cytokine removal may contribute to prevention of intubation in patients infected with COVID-19. Blood Purif. 2021; 50:257–60. https://doi.org/10.1159/000509107 PMID:32594085

18. Fominskiy EV, Scandroglio AM, Monti G, Calabrò MG, Landoni G, Dell'Acqua A, Beretta L, Moizo E, Ravizza A, Monaco F, Campochiaro C, Pieri M, Azzolini ML, et al, and COVID-BioB Study Group. Prevalence, Characteristics, Risk Factors, and Outcomes of Invasively Ventilated COVID-19 Patients with Acute Kidney Injury and Renal Replacement Therapy. Blood Purif. 2021; 50:102–09. https://doi.org/10.1159/000508657 PMID:32659757

19. Jose RJ, Manuel A. COVID-19 cytokine storm: the interplay between inflammation and coagulation. Lancet Respir Med. 2020; 8:e46–47. https://doi.org/10.1016/S2213-2600(20)30216-2 PMID:32353251

20. Liu QQ, Cheng A, Wang Y, Li H, Hu L, Zhao X, Wang T, He F. Cytokines and their relationship with the severity and prognosis of coronavirus disease 2019 (COVID-19): a retrospective cohort study. BMJ Open. 2020; 10:e041471. https://doi.org/10.1136/bmjopen-2020-041471 PMID:33257492

21. Cheng Y, Luo R, Wang K, Zhang M, Wang Z, Dong L, Li J, Yao Y, Ge S, Xu G. Kidney disease is associated with in-hospital death of patients with COVID-19. Kidney Int. 2020; 97:829–38. https://doi.org/10.1016/j.kint.2020.03.005 PMID:32247631

22. Wrapp D, Wang N, Corbett KS, Goldsmith JA, Hsieh CL, Abiona O, Graham BS, McLellan JS. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020; 367:1260–63. https://doi.org/10.1126/science.abb2507 PMID:32075877

23. Li Z. Caution on kidney dysfunctions of COVID-19 patients. 2020. https://doi.org/10.2139/ssrn.3559601

24. Darmon M, Schortgen F, Leon R, Moutereau S, Mayaux J, Di Marco F, Devaquet J, Brun-Buisson C, Brochard L. Impact of mild hypoxemia on renal function and renal resistive index during mechanical ventilation. Intensive Care Med. 2009; 35:1031–38. https://doi.org/10.1007/s00134-008-1372-5 PMID:19242676

25. Gabarre P, Dumas G, Dupont T, Darmon M, Azoulay E, Zafrani L. Acute kidney injury in critically ill patients with COVID-19. Intensive Care Med. 2020; 46:1339–48. https://doi.org/10.1007/s00134-020-06153-9 PMID:32533197

26. Ritchie AI, Singanayagam A. Immunosuppression for hyperinflammation in COVID-19: a double-edged sword? Lancet. 2020; 395:1111. https://doi.org/10.1016/S0140-6736(20)30691-7 PMID:32220278

27. Chan KW, Wong VT, Tang SC. COVID-19: An Update on the Epidemiological, Clinical, Preventive and Therapeutic Evidence and Guidelines of Integrative Chinese-Western Medicine for the Management of 2019 Novel Coronavirus Disease. Am J Chin Med. 2020; 48:737–62. https://doi.org/10.1142/S0192415X20500378 PMID:32164424

28. Kellum JA. Kidney disease: improving global outcomes (KDIGO) acute kidney injury work group. KDIGO clinical practice guideline for acute kidney injury. Kidney Int Suppl. 2012; 2:1–138.

Editorial

Aging and monocyte immunometabolism in COVID-19

Brandt D. Pence

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the third highly pathogenic coronavirus to emerge in the 21st century. The virus, which causes coronavirus disease 2019 (COVID-19), was the first of these coronaviruses to cause a worldwide pandemic, and to date has resulted in over 100 million cases and over 2 million deaths worldwide and has massively impacted the economy of nearly every country. COVID-19 disproportionately affects older adults, with individuals age 80 or older having >20-fold and >300-fold risk of death from COVID-19 compared to 50-59 and 18-39 age groups respectively [1]. Immune dysfunction during aging is well known, and a variety of age-related immune system impairments are thought to impact the response to SARS-CoV-2 in older adults [2]. However, the precise mechanisms by which aging and immunity interact to exacerbate COVID-19 have not been adequately described.

Previously, I suggested a principal role for monocytes in mediating severe COVID-19 in older adults [3]. This argument was predicated on several observations by multiple groups during the early stages of the pandemic. Namely, severe COVID-19 was associated with lung and peripheral tissue infiltration of monocytes and monocyte-derived macrophages, and these cell types also displayed evidence of hyperinflammation and disease-related phenotypic variation which was qualitatively similar to (although more substantial than) changes which occur during the aging process. A number of additional studies have since supported these initial observations. However, molecular mechanisms governing the monocyte response to SARS-CoV-2 are not yet well-characterized, and the interaction between aging and these responses has yet to be described.

In 2018, my laboratory published a short report demonstrating mitochondrial dysfunction in monocytes isolated from older adults [4]. This finding was based on a substantial reduction in maximal respiratory capacity and spare capacity in purified classical monocytes from individuals 60-80 years of age compared to study participants 18-35 years of age. More recently, Saare et al. [5] substantially expanded on our initial work, replicating the observation of mitochondrial dysfunction, and additionally demonstrating increased glucose uptake and altered arachidonic acid metabolism which are indicative of increased basal inflammation in monocytes. These findings support earlier evidence of increased inflammation and cellular dysfunction in aged monocytes [6], suggesting that metabolic changes in the innate immune system related to aging underly (at least in part) the pro-inflammatory state commonly referred to as inflammaging.

Although the molecular basis of the monocyte response to SARS-CoV-2 has yet to be fully described, a few studies have suggested that direct infection of monocytes with the virus initiates metabolic reprogramming indicative of a pro-inflammatory response. Isolated human monocytes respond to SARS-CoV-2 stimulation *in vitro* by upregulating hypoxia-inducible factor (HIF)-1α mediated glycolysis [7], and suppression of this response by pre-treatment of these cells with 2-deoxyglucose (to inhibit glycolysis) or BAY-87-2243 (to inhibit HIF-1α) abrogates the pro-inflammatory and pro-oxidant responses of monocytes to SARS-CoV-2 infection. Likewise, SARS-CoV-2 suppresses mitochondrial respiratory capacity in isolated monocytes [7].

It was also recently observed that monocytes infected with SARS-CoV-2 accumulate intracellular lipid droplets, and that inhibition of this process blocks production of pro-inflammatory cytokines including IL-6, IL-8, and TNFα during viral infection [8]. While the accumulation of intracellular lipids was primarily attributed to increased lipid uptake and triacylglycerol synthesis in infected monocytes, it may also be reflective of impaired fatty acid oxidation by mitochondria in these cells.

Given that SARS-CoV-2 appears to reprogram metabolism in monocytes to promote glucose metabolism and downregulate fatty acid oxidation, it stands to reason that cells which have pre-existing deficits in mitochondrial function (such as monocytes from older individuals) may display exacerbated or aberrant responses to this novel virus. Therefore, age-related metabolic dysfunction in the innate immune system may predispose older individuals to worsened outcomes during COVID-19, contributing to the disproportionate severity of disease in this population. While speculative at this time, these links suggest a therapeutic strategy of immunometabolic modulation may be useful in COVID-19-associated inflammation.

Many common geroprotector drugs – including metformin and rapamycin – are also potent regulators of glucose metabolism and therefore have the potential to be repurposed for the treatment of hyperinflammation during COVID-19.

REFERENCES

1. Williamson EJ, et al. Nature. 2020; 584:430–36. https://doi.org/10.1038/s41586-020-2521-4 PMID:32640463
2. Bajaj V, et al. Front Physiol. 2021; 11:571416. https://doi.org/10.3389/fphys.2020.571416 PMID:33510644
3. Pence BD. Geroscience. 2020; 42:1051–61. https://doi.org/10.1007/s11357-020-00213-0 PMID:32556942
4. Pence BD, Yarbro JR. Exp Gerontol. 2018; 108:112–17. https://doi.org/10.1016/j.exger.2018.04.008 PMID:29655929
5. Saare M, et al. Aging Cell. 2020; 19:e13127. https://doi.org/10.1111/acel.13127 PMID:32107839
6. Hearps AC, et al. Aging Cell. 2012; 11:867–75. https://doi.org/10.1111/j.1474-9726.2012.00851.x PMID:22708967
7. Codo AC, et al. Cell Metab. 2020; 32:437–446.e5. https://doi.org/10.1016/j.cmet.2020.07.007 PMID:32697943
8. da Silva Gomes Dias S, et al. PLoS Pathog. 2020; 16:e1009127. https://doi.org/10.1371/journal.ppat.1009127 PMID:33326472

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Editorial

Evaluation of long-term COVID-19

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COVID-19 late complications. Coronavirus disease 2019 (COVID-19), which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has the potential to become a long-term health problem due to persistent symptoms that arise following the acute viral infection. Many individuals who have COVID-19 make a full recovery and return to their baseline state of health. Others, however, have symptoms or other sequelae for weeks and months after initial SARS-CoV-2 infection. The constellation of symptoms and other effects experienced by patients who do not return to their baseline state of health after COVID-19 has been referred to by many names, including post-acute sequelae of COVID-19 (PASC), post-COVID condition or syndrome, and long or long-haul COVID.

Although COVID-19 initially affects the lungs, clinical and scientific evidence is evolving regarding the long-term effects of COVID-19, which can be wide-ranging in severity and duration and can affect multiple organ systems, with symptoms such as fatigue, dyspnea, cognitive dysfunction, anxiety, and depression often described. Longer term effects have been reported in all age groups and demographics and in people with asymptomatic, mild, or severe COVID-19 illness.

Emerging studies indicate a broad organotropism of SARS-CoV-2 beyond the lungs to other tissues, organs, and systems resulting in multiorgan dysfunction, such as renal complications, gastrointestinal dysfunctions, endocrine system disorders, thromboinflammation, neurological dysfunctions, dermatological symptoms, hematological manifestations, and myocardial dysfunction and arrhythmia. Cellular damage due to a robust innate immune response with cytokine production and a pro-coagulant state induced by SARS-Co-V-2 may contribute to these sequelae [1]. This broad effect of COVID-19 disease is consistent with the finding that SARS-CoV-2 gains entry to cells by binding to the cell surface receptor angiotensin-converting enzyme-2 (ACE-2), which is widely distributed on many cell types in human tissues.

Many recent studies have reported on the long-term effects of COVID-19 (see Nalbandian review [1]). Of note is a study that quantified the rates of organ dysfunction in individuals with COVID-19 after discharge from the hospital compared with a matched control group from the general population [2]. The research team from University College London tracked rates of hospital readmission in over 47,000 COVID-19 patients and the control group for all causes of mortality and diagnoses of respiratory, cardiovascular, metabolic, kidney, and liver diseases. Over a mean follow-up time of 140 days, nearly one-third of individuals who were discharged from the hospital after acute COVID-19 were readmitted and more than 10% died after discharge, with these events occurring at rates 4 and 8 times greater than the matched control group, respectively. Rates of respiratory disease, diabetes, and cardiovascular disease were also significantly higher in COVID-19 patients. The researchers concluded that patients discharged from the hospital after COVID-19 had increased rates of multiorgan dysfunction compared with the expected risk in the general population. Importantly, they suggested that urgent research is needed to establish the risk factors.

Another recent research article highlighted the persistent neurological and cognitive dysfunction-related sequelae associated with non-hospitalized, COVID-19-infected individuals termed “long-haulers” [3]. The main neurological symptoms were “brain fog” (81%), headache (68%), numbness/tingling (60%), dysgeusia (59%), anosmia (55%), and myalgia (55%).

The expansive global burden of SARS-CoV-2 infection suggests that the potential public health effects of post-acute COVID-19 are significant, even if a small proportion of infected persons have prolonged recovery or fail to return to their baseline state of health. Specialty clinics have been established both in the United States and worldwide to address the increasing need to care for these COVID-19 patients. At a recent NIH workshop on post-acute COVID-19, clinicians provided observations on the diverse needs of patients, which included multisystem symptoms (such as fatigue, mental health problems, and pain) and other signs and symptoms pointing to specific organ systems (such as renal, cardiac, pulmonary, and gastrointestinal). These patients will require individualized and multi-disciplinary approaches to treatment [4], and clearly a simple, non-invasive test to diagnose early injury to various organs and to monitor progression and treatment of disease in these patients would be extremely useful.

Universal Screening Test (UST). Predictions or early detection of potential COVID sequelae would be very

useful for disease and treatment monitoring and could be quite helpful if detected before discharge from the hospital. However, as described above, there are numerous potential late complications of COVID infection involving different organs, and their manifestations may vary from subject to subject. Thus, screening for early detection of various organ pathologies would be complicated and expensive. Recently, we proposed the development of a Universal Screening Test (UST) for early detection of potential pathologies of various organs and tissues [5]. The UST concept is based on analysis of cell-free microRNA (miRNA) signatures circulating in the blood plasma. miRNAs are short, non-coding regulatory molecules that have several advantages as potential biomarkers: (i) many of over 2000 known human miRNAs are specific to or highly enriched in particular organs, tissues, and cell types, and hence, changes in their plasma concentrations should reflect physiological and pathological processes in corresponding organs, tissues, or cell types; (ii) such miRNAs can be detected with high specificity and sensitivity in one multiplex molecular panel; and (iii) miRNAs have relatively high stability in circulation as compared with other RNA species.

The concentration of individual miRNAs in plasma depends on a number of factors, including levels of synthesis in different cells and secretion/excretion, cell death, degradation of cellular compartments (e.g., synapsis and neurites in neuronal cells), and structural form in extracellular space (such as exosomes, microvesicles, or complexed with proteins and lipids). In addition, plasma concentrations of miRNAs enriched in particular organs may be affected by changes in blood supply caused by aging-related processes, tumor growth, and other factors. Thus, in order for plasma miRNAs to be useful as biomarkers, these factors must be taken into consideration. To compensate for these variations, we have successfully used miRNA pairs (a ratio between two miRNAs) instead of individual miRNAs as pathology biomarkers [5-7]. Biomarker pairs in which miRNAs are highly correlated have proven to be most effective [5]. Our studies demonstrated that three to four miRNA pairs can be combined into an miRNA classifier to increase the overall sensitivity. The feasibility of disease detection using the UST approach has been tested in pathologies of several organ systems based on the analysis of circulating organ-enriched miRNAs: (a) neuro-degeneration in different brain regions due to Alzheimer's disease, Parkinson's disease, fronto-temporal dementia, and amyotrophic lateral sclerosis [5,6]; (b) Rett syndrome [7], a neurodevelopmental disorder that can cause dysfunction of different organs; (c) lung pathologies, such as pneumonia, asthma, and early stages of cancer [5]; and (d) cancers of the gastrointestinal system (esophagus, stomach, colon), as well as Crohn's disease [5]. The ability of UST to detect organ damage in a wide variety of diseases suggests that it will be generally applicable to detecting post-acute COVID pathologies without limitations to the organs and tissues affected.

Summary. Based on our findings, we strongly believe that the UST approach can be useful for prediction, early detection, and monitoring of COVID-19 sequelae. All plasma miRNAs necessary for the analysis of pathology in different organs can be detected in one assay, which makes it not only clinically relevant but also much more efficient than multiple assays for different organs. Further, analysis of epigenetic miRNA biomarkers reflective of underlying pathophysiological processes can lead to better understanding of the pathology.

REFERENCES

1. Nalbandian A, et al. Nat Med. 2021; 27:601–15. https://doi.org/10.1038/s41591-021-01283-z PMID:33753937
2. Ayoubkhani D, et al. BMJ 2021; 372:n693 https://doi.org/10.1136/bmj.n693 PMID:33789877
3. Graham EL, et al. Ann Clin Transl Neurol. 2021; 8:1073–85. https://doi.org/10.1002/acn3.51350 PMID:33755344
4. Lerner AM, et al. Ann Intern Med. 2021; M21-1043. https://doi.org/10.7326/M21-1043 PMID:33780290
5. Sheinerman KS, Umansky S. Expert Rev Mol Diagn. 2015; 15:329–38. https://doi.org/10.1586/14737159.2014.973858 PMID:25354444
6. Sheinerman KS, et al. Alzheimers Res Ther. 2017; 9:89. https://doi.org/10.1186/s13195-017-0316-0 PMID:29121998
7. Sheinerman K, et al. PLoS One. 2019; 14:e0218623. https://doi.org/10.1371/journal.pone.0218623 PMID:31291284

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Review

Cellular senescence in lymphoid organs and immunosenescence

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ABSTRACT

Immunosenescence is a multi-faceted phenomenon at the root of age-associated immune dysfunction. It can lead to an array of pathological conditions, including but not limited to a decreased capability to surveil and clear senescent cells (SnCs) and cancerous cells, an increased autoimmune response leading to tissue damage, a reduced ability to tackle pathogens, and a decreased competence to illicit a robust response to vaccination. Cellular senescence is a phenomenon by which oncogene-activated, stressed or damaged cells undergo a stable cell cycle arrest. Failure to efficiently clear SnCs results in their accumulation in an organism as it ages. SnCs actively secrete a myriad of molecules, collectively called senescence-associated secretory phenotype (SASP), which are factors that cause dysfunction in the neighboring tissue. Though both cellular senescence and immunosenescence have been studied extensively and implicated in various pathologies, their relationship has not been greatly explored. In the wake of an ongoing pandemic (COVID-19) that disproportionately affects the elderly, immunosenescence as a function of age has become a topic of great importance. The goal of this review is to explore the role of cellular senescence in age-associated lymphoid organ dysfunction and immunosenescence, and provide a framework to explore therapies to rejuvenate the aged immune system.

INTRODUCTION

The aging population

Aging is the gradual process of organismal deterioration which is associated with a multitude of age-related disorders and diseases that make one wonder if aging itself is a disease that needs to be addressed [1]. A shadow is cast on the benefits of longevity if the elderly are faced with the possibility of a decline in their quality of life. The world currently has over 700 million people who are over the age of 65, a number that is projected to grow rapidly in the near future [2]. As advancing age is strongly correlated to decreased quality of life and increased risk of several age-related diseases [3], these demographics seem more dismal in prospering countries, with the USA and the UK having about 16–18% of their population over the age of 65 [4, 5]. With the life expectancy of most Western countries steadily increasing, majority of people are expected to spend at least 2 decades, or 25% of their life, over the age of 65, when they are prone to acquiring various age-related morbidities [6, 7]. The silver lining to this otherwise tragic situation is that results from recent studies indicate that the aging process and the pace of organismal deterioration is malleable and can be influenced greatly by physiological, genetic, dietary and pharmaceutical interventions [8–16].

The aging immune system

The immune system is a complex network of cells and tissues working in coalition to maintain the health of an organism. It not only clears foreign pathogens, but also helps to maintain the integrity of the organism by clearing away dead or dysfunctional cells [17–22]. Due to the immune system's complexity and intricacy, 7% of the genes from the human genome are allocated exclusively for its functioning and maintenance [23].

Like any other system, the immune system changes with age and experiences gradual deterioration. Improving our understanding of this phenomenon is of great significance because the medical and scientific advancements that have facilitated the unprecedented increase in average human lifespan have been unable to significantly increase the human healthspan [24]. Because of this, we have a rapidly increasing aging population in a world where there is a substantial risk of steep decline in quality of life with age.

Age-associated deterioration and dysfunction of the immune system leads to the establishment of an incompetent immune response against invading pathogens [25, 26]. This could partially provide an explanation for the age-dependent increase of mortality in patients suffering from infections like influenza [27], with people older than 65 accounting for more than 90% of the influenza-associated annual deaths [28]. Furthermore, the aged immune system elicits an inadequate response to vaccines, leaving the elderly susceptible to pathogens despite being vaccinated against them [29, 30]. This is especially poignant in the wake of an ongoing pandemic where the mortality rate is disproportionately high in the elderly [31].

Aging of the immune system is also one of the major factors that accelerates the deterioration of an organism, as its dysfunction not only fails to elicit a strong immune response against invading pathogens but also drives the accumulation of undesirable and malfunctioning cells [25, 32–36]. In some cases, the aging immune system also develops an affinity for attacking self-antigens, leading to autoimmunity-associated disorders [37, 38].

In recent years, there have been many studies that have broadened our understanding of the aging immune system and immunosenescence (the gradual deterioration of the immune system with age) from the perspective of genetics, nutrition, physiology, and molecular biology [39–42]. Despite this assimilation of knowledge, a complete understanding of the dynamics of this process is lacking.

Within a systemic context, the age-related changes and adversities in any organ system arise from a complex crosstalk between different cells and processes of the body. By virtue of the way that research studies are designed and funded, many aspects of this complexity are often overlooked. In this review, we will discuss one such interaction, between cellular senescence and the immune system with a focus on the accumulation of SnCs in the lymphoid organs of the aging body, which is greatly understudied and underappreciated.

Cellular senescence

Initially described in 1961, cellular senescence is the phenomenon by which cells cease to divide despite the availability of adequate growth factors [43]. It was later established that upon encountering certain types of stress and irreparable damage, cells tend to enter a stable cell cycle arrest [44]. From an evolutionary perspective, this is widely considered to be a protective mechanism to prevent the stressed and damaged cells from becoming deleterious to the body.

Like most things optimized by evolution, cellular senescence is not of much concern to the younger body capable of reproduction while the older body, past its reproductive prime, is adversely affected by it. The fitness benefits that cellular senescence provides to younger, reproductively active animals, such as preventing cancer [45], mitigating the progression of fibrosis [46–48] and promoting optimal wound healing [49], have helped the phenomenon survive the arduous tests of natural selection over the millennia. Unfortunately, in almost an antagonistically pleiotropic manner, accumulation of SnCs is very detrimental to the older body [50]. Specifically, SnCs secrete various factors classified together as senescence-associated secretory phenotype (SASP) which cause instability and dysfunction in their surrounding environment [51]. Both SnCs and SASP factors have been implicated in many of the age-related deteriorations, dysfunctions and diseases including but not limited to frailty, hypertrophy of tissue, stem-cell exhaustion, bystander effect mediated senescent cell accumulation, and cancer [51–63].

The interactions between SnCs and the immune system run in both directions, with the immune system surveilling and clearing the SnCs; while the SnCs frequently impede the function, and in some contexts, generation of immune cells. In young and healthy individuals, the immune system can rapidly clear SnCs after their induction, which prevents them from significantly accumulating and causing adverse effects [18, 64]. In older individuals, this turnover is slow and leads to the accumulation of SnCs [34]. Ovadya et al. demonstrated that accumulation of SnCs is accelerated

upon impaired immune surveillance [32]. Since advancing age is associated with impairment in immune function [65], the decline in the turnover of SnCs with age can, at least partially, be attributed to this impediment. Despite multiple studies demonstrating various mechanisms via which SnCs could evade immune clearance [66, 67], the impact of aging on immune evasion of SnCs is not yet completely understood. Of note, SnCs have been shown to cause stem cell exhaustion and dysfunction [62, 68–72]. This is of great relevance and importance to the topic of immunosenescence because senescence, exhaustion and dysfunction of hematopoietic stem cells (HSCs), causes myeloid skewing and a decrease in the production of immune cells which may be one of the underlying causes of age-related immunosenescence.

With many more possible domains of interaction between cellular senescence and the immune system, as seen in (Figure 1), this review will discuss literature that states or suggests the presence of this interaction, with a focus on cellular senescence in the lymphoid organs, and raises questions that need to be answered to strengthen the foundation of the role of cellular senescence in immunosenescence.

CELLULAR SENESCENCE IN THE ORGANS OF THE IMMUNE SYSTEM

Bone marrow

Bone marrow is a spongy tissue residing in the core of vertebrae, skull and long bones. It is the home of HSCs

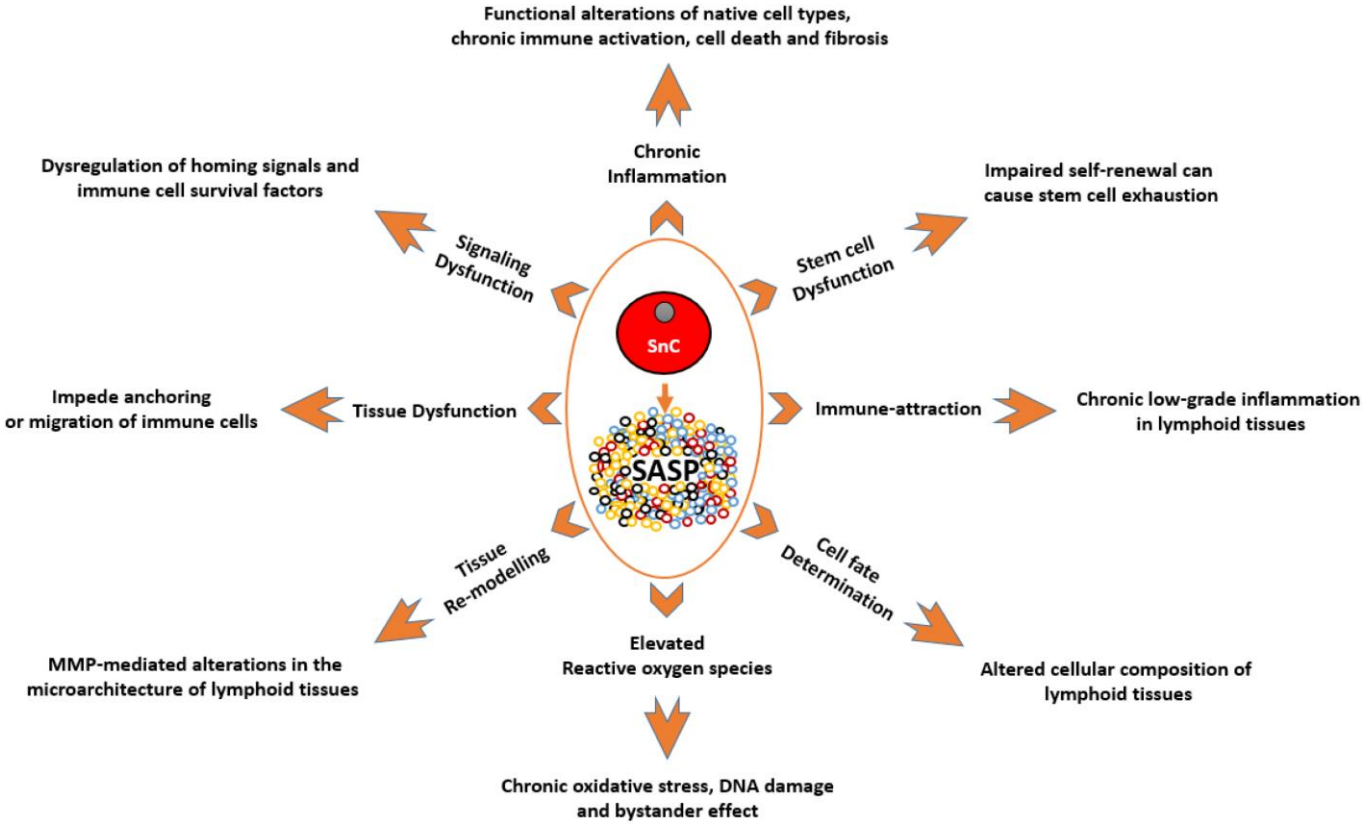

Figure 1. A depiction of the known effects of SnCs and SASP on different cell types and tissues, and how they are relevant to the immune system. SnCs possess altered morphology and surface markers and usually fail to perform the tasks of their non-senescence counterparts. This makes them the dysfunctional units of a tissue which can impede normal functions such as, immune cell priming and transmigration. MMPs produced by SnCs can modify the surrounding matrix and alter the microarchitecture of the lymphoid organs. As these organs are precisely organized into zones with specialized functions, such micro-architectural alterations can lead to dysfunction. SASP produced by SnCs can act as a chemoattractant to immune cells which can lead to unresolved chronic inflammation in tissues. SASP by itself can be inflammatory which can adversely impact neighboring cells. This chronic unresolved inflammation can lead to pathological conditions like fibrosis and neoplasia. SASP-mediated signaling and ROS-mediated oxidative stress can impair clonogenicity and functionality of HSCs, immune cells and other supporting cells of the immune system. SnCs and SASP can alter the expression profile of supporting cells leading to the dysregulation of homing signals required for proper localization of immune cells, and survival factors required for the endurance of certain immune cells. SnCs, by means of SASP, can influence the cell fate of differentiating cells and in some cases, cause the accumulation of adipocytes in the lymphoid organs. Abbreviations: SnC: Senescent cell; SASP: Senescence associated secretory phenotype; MMPs: Matrix metalloproteases; ROS: Reactive Oxygen Species; HSC: Hematopoietic stem cell.

which give rise to most of the immune cells [73]. HSCs are self-renewing pluripotent cells that can generate the entire hematopoietic system.

With increasing age, the bone marrow microenvironment changes dramatically. With advancing age, HSC number increases, while their functionality, including self-renewal and clonogenicity declines. These changes are accompanied by myeloid skewing, elevated adipogenesis in the bone marrow, and alterations in the bone marrow niche [74–78]. Along with the prevalence of significantly more apoptotic cells, bone marrow cellularity (volume occupied by HSCs) decreases significantly with age reaching values lower than 40% [79]. A graphic depiction of the aged bone marrow microenvironment is illustrated in (Figure 2).

Myeloid skewing of HSCs with aging may be in part attributable to the aged bone marrow microenvironment, as even young HSCs develop a myeloid bias upon being transplanted into old mice [80, 81]. It has been suggested that chemokine ligand 5 (CCL5) is a major factor that drives myeloid skewing of HSCs with advancing age. Over expression of CCL5 causes a decrease in pro-lymphoid transcription factors and T-cell differentiation, while genetically knocking out CCL5 prevents myeloid skewing in mice [82]. Age-related accumulation of adipocytes in the bone marrow has been attributed to the increased expression of receptor activator of nuclear factor kappa-B ligand (RANKL) [83]. These bone marrow adipocytes in-turn produce an array of factors that have been shown to affect hematopoiesis and skew it towards myeloid lineage [84–88].

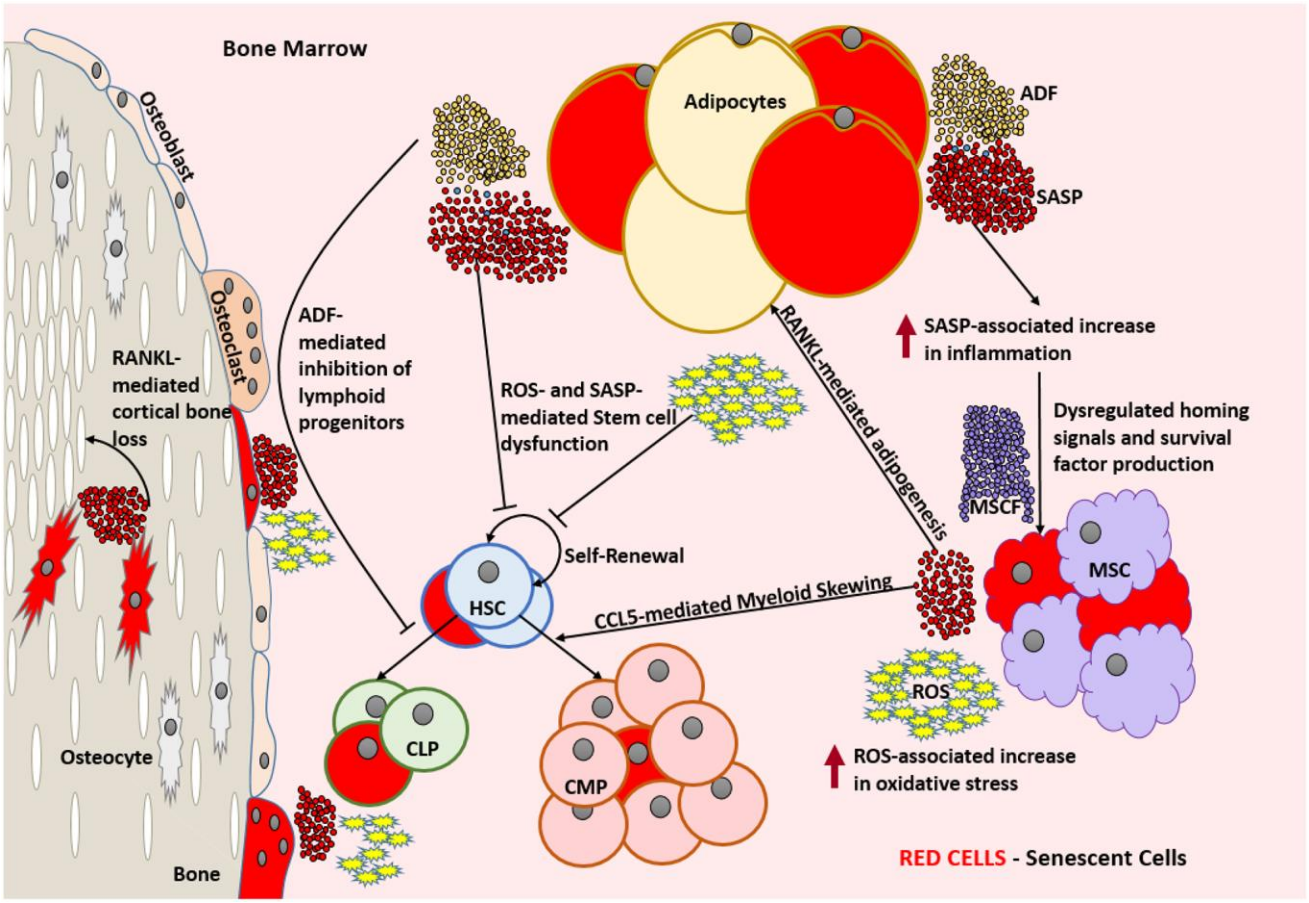

Figure 2. Aged bone marrow microenvironment with accumulated SnCs is not conducive for its normal functionality. SASP and ROS mediate dysfunction and DNA damage in HSCs, respectively and lead to a change in the HSC repertoire and exhaustion of the functional HSC reservoir. RANKL mediates the accumulation of adipocytes that produce ADFs. CCL5 and ADFs mediate the establishment of myeloid skewing in HSCs. SASP mediated inflammation can dysregulate the adequate production of homing signals and survival factors by the MSCs which can lead to the depletion of selective immune cell types. The increased ROS and SASP mediated inflammation causes damage to the surrounding cells and induces senescence by means of the bystander effect. SnCs such as osteocytes can produce SASP that is detrimental to the bone housing which encloses them. In the absence of rapid clearance of SnCs, this becomes a self-perpetuating cycle of dysfunction and damage causing severe immunosenescence. Abbreviations: SnC: Senescent cell; SASP: Senescence associated secretory phenotype; ROS: Reactive Oxygen Species; HSC: Hematopoietic stem cell; CLP: Common lymphoid progenitor; CMP: Common myeloid progenitor; MSC: Mesenchymal stem cell; MCSF: Mesenchymal stem cell derived factors; ADF: Adipocyte derived factors; CCL5: Chemokine Ligand 5; RANKL: Receptor activator of nuclear factor kappa-B ligand.

The accumulation of various $p16^{INK4a}$ positive cells [89–91], SASP factor (like CCL5 and RANKL) generating cells [91–94] in aged bone marrow, along with increased number of cells harboring DNA damage and elevated ROS [95, 96], suggests that age-dependent bone marrow changes can be in part attributed to the accumulation of SnCs.

Based on data showing that the expression profile of adipocytes resembles the SASP profile of SnCs [97], it is likely that a great proportion of these adipocytes are senescent. This became evident after a study where clearance of SnCs in INK-ATTAC mice, a genetically altered model that clears cells expressing $p16^{INK4a}$, showed a significant reduction in the number, size, and tissue volume of bone marrow adipocytes [98]. Other studies have also shown that, despite the structural and functional support provided by adipocytes, they adversely influence the hematopoietic environment [99, 100]. However, whether this is completely attributable to senescent adipocytes and their SASP is yet to be determined.

A recent study implicated the senescence of bone marrow-derived mesenchymal stem cells (BM-MSCs) in the age-associated dysfunction of HSCs, in humans. This study revealed that a significantly higher portion of senescent MSCs were seen in the bone marrow explants of the elderly when compared to their younger cohorts. This was established by showing increased accumulation of cells with DNA damage, elevated ROS and SASP expression. They also showed that the functionality and clonogenicity of young HSCs were impaired when exposed to factors generated by these MSCs [95]. The inflammatory environment, created by SASP of these SnCs, can alter the expression profile of normal MSCs to dysregulate the expression of factors necessary for lymphocyte survival [101–105].

Along with the cell-extrinsic causes for stem cell aging, older HSCs show an accumulation of senescence in association with increased DNA damage and telomere attrition, along with having an increased risk of undergoing an inflammatory cell death known as pyroptosis [68, 106]. Reactive oxygen species (ROS) produced by SnCs play a key role in the bystander effect [107]. ROS produced by SnCs in the bone marrow environment can cause DNA breaks in HSCs. This agrees with the finding that aged HSCs harbor more DNA damage compared to their younger counterparts [108]. As the DNA damage repair mechanism is not robust and quite error prone in the quiescent HSCs [109], the constant oxidative stress-induced DNA damage can progressively deplete and alter the functional HSC repertoire with increasing age [110].

Direct evidence for the adverse role of cellular senescence in modulating HSC function during aging was provided by demonstrating that knocking out $p16^{INK4a}$ conserved HSC functionality and stress tolerance with age [68]. A more recent study from our lab has shown that clearing SnCs rejuvenated the aging HSC repertoire by reducing myeloid skewing and improving clonogenicity significantly in mice [63].

Thymus

The thymus is a primary lymphoid organ located behind the breastbone and above the heart, within which T-cells mature. In an evolutionarily conserved manner, most vertebrates experience an age-associated thymic involution, which is characterized by atrophy and the development of cavities. An age-dependent alteration in thymic cellularity can be seen, with most functional cells getting replaced by fibroblasts, fat cells and senescent cells [111–116].

Thymic atrophy is associated with the reduced turnover of new T-cells [117], a constricted T-cell receptor repertoire [118] and the production of higher autoreactive T-cells that could lead to autoimmunity [119]. As depicted in (Figure 3) these are characteristic features of immunosenescence that play an important role in age-associated impaired T-cell function [120].

Thymic epithelial cells (TECs) from adult human thymus stained positive for senescence- associated beta galactosidase (SA-βGal) and the thymic tissues from these adults also strongly stained positive for markers of oxidative DNA damage such as γH2AX and 8-oxoguanine [121]. A similar finding of high γH2AX staining was seen in the thymus of old mice, which was indicative of DNA damage and cellular senescence [111]. This also correlated with the increased inflammatory environment of the aged thymus seen in humans [122]. Despite the abundance of evidence suggesting accumulation of SnCs in atrophied thymus, whether cellular senescence plays a causal role in thymic involution needs to be further studied, as the accumulation of SnCs could be a consequence of thymic involution. But the possibility of a causal involvement of SnCs and their SASP seems likely because the administration of IL-6, a known SASP factor, has been shown to induce thymic atrophy [122]. In addition, increased oxidative stress and DNA damage in the stromal cells, especially TECs, has also been shown to accelerate thymic aging [123].

With the existing knowledge that TECs play a crucial role in the positive and negative selection of maturing T-cells [124], the role of senescent TECs in the thymic environment should also be explored in the

context of positive and negative selection of T-cells. For example, it has yet to be determined whether the interaction of the developing T-cells with SnCs of the thymus play a role in the development of T-cells with auto-reactivity.

Interestingly, the recruitment of T-cell progenitors to the thymus is similar between young and old mice [125]. The reduced T-cell output has been attributed to the defective microenvironment of the thymus and other secondary lymphoid organs [125–127]. Though there is a significant functional decline in thymic activity, the aged thymus still retains a portion of its function [128], which leads us to believe that the therapeutic clearance of SnCs could help to restore thymic function in the elderly. Thymic regeneration strategies so far have largely failed to improve the production of functional of T-cells, in part due to the lack of a systemic approach, because rejuvenating the thymus alone still leaves the secondary lymphoid organs too impaired to support the naïve T-cells being produced [127, 129].

It would be intriguing to replicate these studies with a senolytic combinatorial therapy to see how it changes the outcome. It should be a promising venture, because caloric restriction, a dietary intervention known to reduce cellular senescence [130] and SASP [131, 132], has been shown to delay thymic involution and mitigate thymic adipogenesis [133].

Spleen

The spleen is a secondary lymphoid organ that acts as a blood filter to remove damaged red blood cells. It plays a crucial role in maintaining the optimal populations of white blood cells and platelets. The spleen can detect pathogenic invaders in the blood and mobilize the immune system to fight against the pathogens [134].

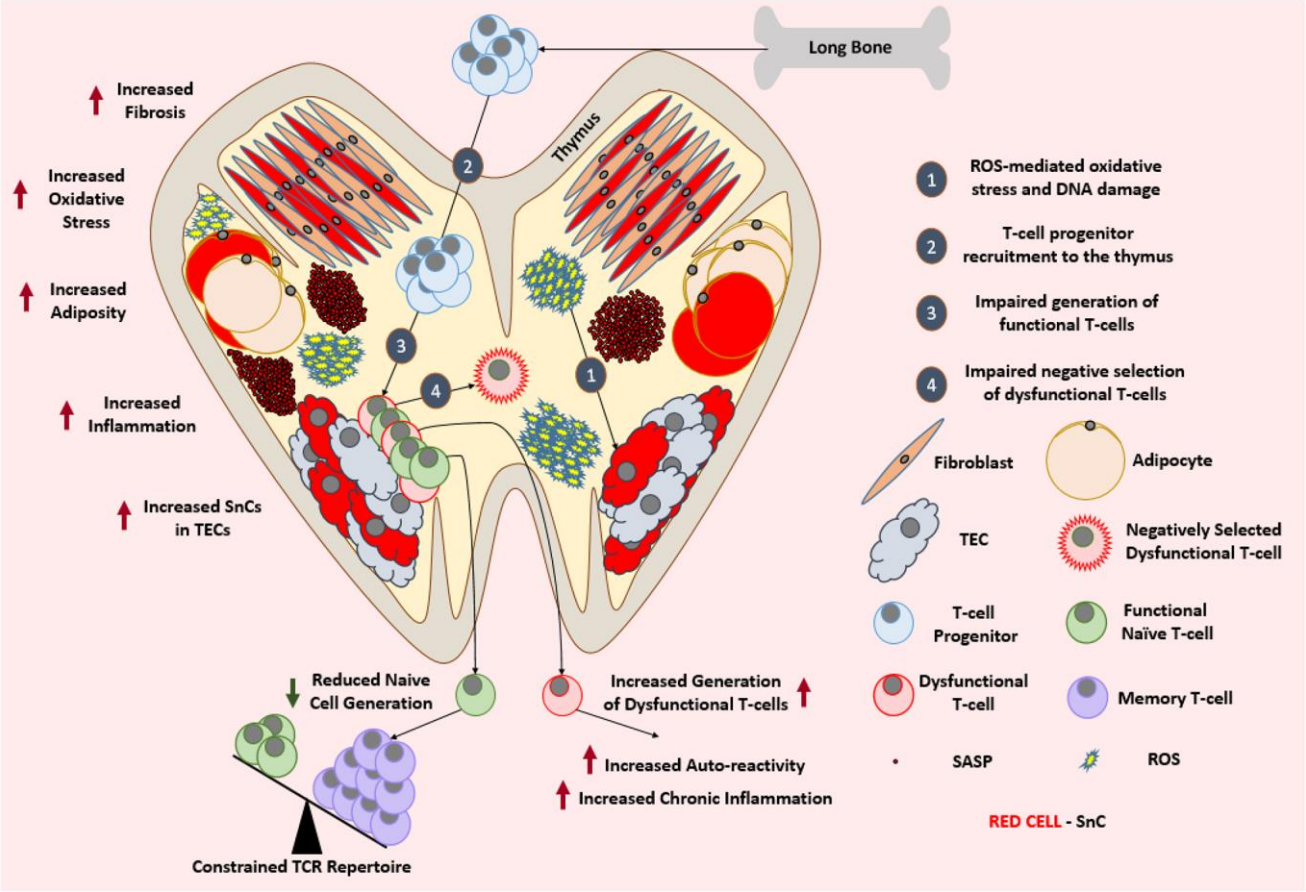

Figure 3. Aged thymus is dysfunctional. With advancing age, thymus loses its cellularity while accumulating adipocytes and fibroblasts. Aged thymus develops an inflammatory environment with high levels of oxidative stress. This is evident by the accumulation of senescent TECs with elevated markers of DNA damage and oxidative stress. Despite the adequate recruitment of T-cell progenitors, aged thymus generates inadequate number of naïve T-cells which leads to the age-associated depletion of TCR repertoire and ultimately a change in the immune cell landscape. Due to the impaired negative selection of dysfunctional T-cells, the aged thymus shows an increase in the output of dysfunctional and autoreactive T-cells leading to the establishment of low-grade chronic inflammation. Abbreviations: SnC: Senescent cell; SASP: Senescence associated secretory phenotype; ROS: Reactive Oxygen Species; TEC: Thymic epithelial cell; TCR: T-cell receptor.

With advancing age, the cellularity and microarchitecture of the spleen changes significantly accompanied by altered localization of various cells [135]. The distinct demarcation of T-cell and B-cell regions within the white pulp becomes obscure with advancing age. Also, an alteration in the organization and function of stromal cells, marginal zone macrophages and marginal metallophilic macrophages can be seen [136]. An accumulation of SnCs with advancing age has been demonstrated to happen in the spleen. This was shown not only by means of elevated expression of $p16^{INK4a}$ and SASP factors, but also by means of cell accumulation with elevated DNA damage [50, 137]. It has also been shown that the stromal cell populations of the aged spleen, exhibit an upregulated expression of IL-6, a SASP factor, implying that at least a proportion of these cells could be senescent [138].

Age-dependent changes in the splenic microenvironment impair the phagocytic capacity of macrophages in the marginal zone. While the phagocytic capacity of macrophages from the aged spleen seemed to be less efficient *in vivo*, their *in vitro* phagocytic capacity was similar to those from young mice [139]. Interestingly, induction of SnCs accumulation in the spleen after radiation has been shown to impart similar functional impairments to splenic macrophages in mice, and the clearance of such SnCs was able to restore macrophage function [140]. Microenvironment-dependent dysfunction and impaired migration of B-cells can also be seen in the aged spleen [141]. Even B-cells originating from young HSCs in an aged recipient showed signs of dysfunction, providing support to the idea that B-cell dysfunction is mainly attributable to the aged splenic environment [135].

Splenic priming of T-cells is a crucial step in the establishment of an appropriate T-cell response [142]. It is known that the senescent splenic environment impairs the recruitment of T-cells to the spleen. In addition, as depicted in (Figure 4), the microenvironment-mediated impairment of the functionality of antigen-presenting cells such as B-cells, macrophages and dendritic cells (DCs) in the aged spleen may explain why even T-cells originating from young HSCs were dysfunctional and showed a delayed response to stimulation in an aged splenic microenvironment [143, 144].

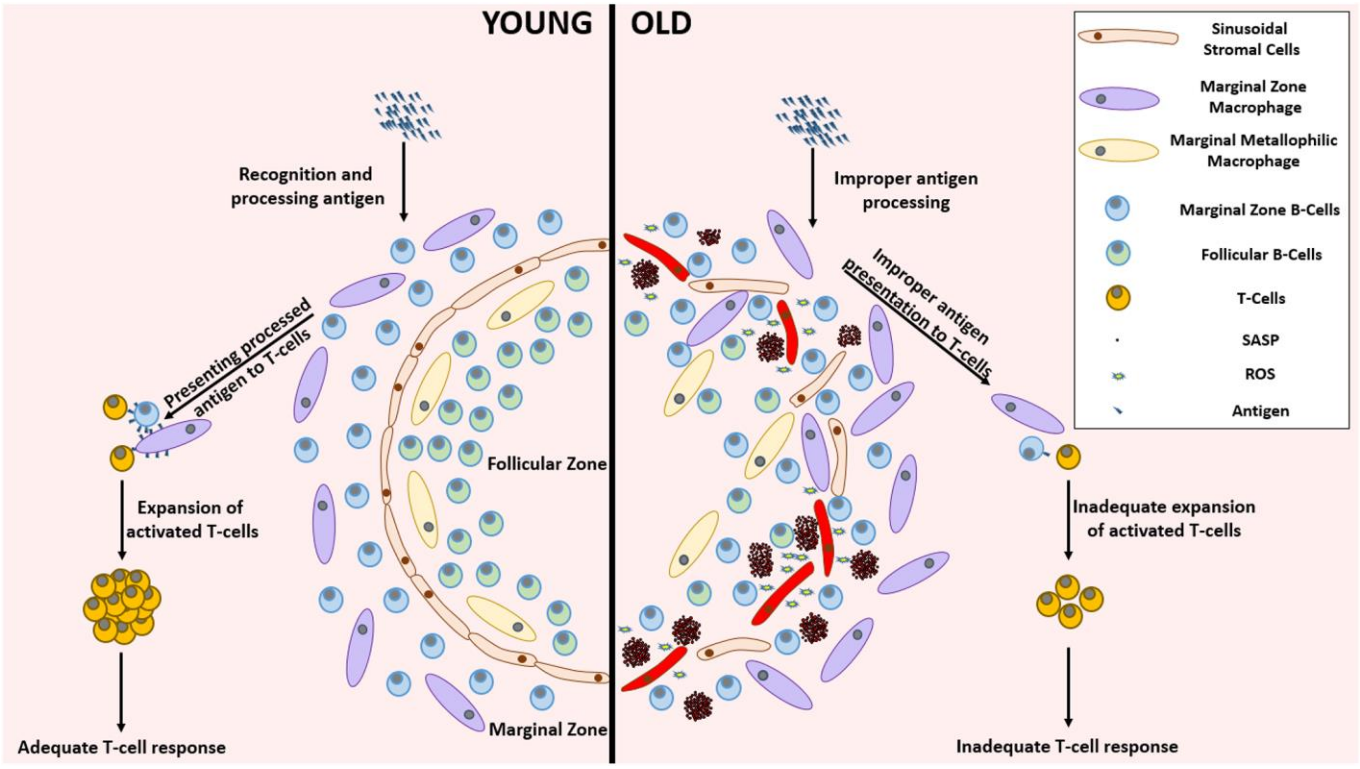

Figure 4. Remarkable differences between the young and aged splenic environment. With advancing age, the stromal cells in the lining of sinuses, that demarcate follicular zone from the marginal zone, become less organized accompanied with an altered localization of various cell types. The inflammatory environment created by the accumulation of SnCs impairs the functionality of several cells residing in the spleen. This functional impairment mediated improper antigen presenting capabilities lead to the establishment of an inadequate T-cell response against pathogenic invasion. Abbreviations: SnC: Senescent cell; SASP: Senescence associated secretory phenotype; ROS: Reactive Oxygen Species.

Though not deeply explored in these studies, it is apparent that the splenic environment of the old mice is not conducive for the proper functionality of various immune cells. Apart from SASP-mediated micro-environmental alterations, SnCs, by virtue of their altered morphology, can imbue structural alterations to the aged spleen. With senolytics [14, 15, 145–148] and senostatics [149] becoming more accessible, further insights into SASP-independent mechanisms of SnCs involvement in immunosenescence should be explored.

Lymph nodes

Lymph nodes are small bulbous structures that form a crucial part of the lymphatic system along with the lymphatic vessels. They filter the lymph fluid obtained from the surrounding tissues before it re-enters the blood stream [150].

Lymph nodes house various immune cells including T-cells, B-cells and DCs and play an essential role in establishing a strong immune response [151–154]. With advancing age, there is a significant decline in the number, integrity, and functionality of lymph nodes [135, 155–158]. Alterations in cellularity and functionality of different cell types of lymph nodes have been shown to occur with advancing age (reviewed here [158]). Increased adiposity and fibrosis have also been described in lymph nodes of patients older than 60 years [155, 156].

It has been speculated that lymphatic endothelial cells and high endothelial venules of the lymph nodes show signs of aging similar to that of the vascular system. This includes altered permeability, accumulation of SnCs, and increased inflammation, which could act as causal factors that adversely affect the migration and recruitment of immune cells like naïve T-cells [158]. It has also been shown that the age-dependent increase in the level of prostaglandin-2 in the lungs inhibits the migration of DCs to the draining lymph nodes, leading to the establishment of an improper T-cell response to viral infections like SARS-CoV [159]. This is interesting since prostaglandin production is upregulated in SnCs [160], and provides evidence on how cellular senescence in other organs can indirectly impact the function of lymph nodes.

Stromal cells from aged lymph nodes have reduced replicative potential upon stimulation [161, 162] and were unable to support naïve T-cell homeostasis [127]. Though not explored as a possibility in these studies, this could be an indication that at least a portion of these stromal are senescent. Another interesting study sheds light on the role of chemokine ligand 2 (CCL2) produced by the stromal cells of lymph nodes in the mitigation of antibody response [163]. Despite this being an important function that prevents the establishment of unnecessary germinal centers in the absence of an antigen, CCL2 is a SASP factor, which raises the question of whether senescent stromal cells that perpetually produce CCL2 are responsible for the age-dependent impairment of lymph nodes to support germinal centers [157].

It seems highly likely that cellular senescence is involved in this age-related lymph node deterioration. Further studies exploring the presence of SnCs in the aged lymph nodes and their role in lymph node-mediated immune response are needed.

Mucosa associated lymphoid tissue

Mucosa-associated lymphoid tissue (MALT) is a part of the immune system that localizes on the surface of the mucosal tissues. Depending on their location, MALT is classified into different types, such as inducible bronchus-associated lymphoid tissue (iBALT) [164, 165], conjunctiva-associated lymphoid tissue (CALT) [166, 167], larynx-associated lymphoid tissue (LALT) [168] and inducible skin-associated lymphoid tissue (SALT) [169, 170]. The most commonly studied MALT representatives are nasopharynx-associated lymphoid tissue (NALT) [171, 172] and gut-associated lymphoid tissue (GALT) [173].

In humans, the adenoids of the nasopharynx, tonsils of oropharynx, and a few more lymph nodes in the region form the Waldeyer's ring [174, 175]. They are considered to be a part of the MALT and are analogous to the NALT in rodents [172]. They are crucial for immunization through intranasal vaccination [176]. Similarly, GALT is comprised of Peyer's patches, mesenteric lymph nodes (MLNs) and isolated lymphoid follicles (ILFs) [177].

The MALT functions in a complex manner (reviewed here [178]), which is known to be affected by the process of aging, as seen in mice by the age-dependent reduction in the establishment of oral tolerance to novel antigens [179]. This deterioration varies regionally, with NALT conserving its functionality for longer than GALT, making nasal immunizations an attractive alternative for vaccinating the elderly [180, 181].

Though cellular senescence has been shown to be present in the tonsils of patients with tonsillitis and

tonsillar hypertrophy, it is still unclear whether SnCs play a role in these pathological conditions [182, 183]. Despite knowing that tonsillar mesenchymal stem cells can undergo cellular senescence [184, 185], the implications of cellular senescence in alterations of the function of NALT has not yet been studied.

Extensive studies in mice show that GALT exhibits a similar age-associated alteration in the cellular composition and decline in functionality like many of the other parts of the immune system. There is a decline in naïve T-cell and B-cell repertoires which are primarily replaced by memory cells [186, 187]. An age-dependent impairment in proliferative response to mitogenic stimulus is also seen in GALT [188]. There is a quantitative decline in dendritic cells accompanied by impaired functionality [189, 190] that yields a similarly impaired priming of T-cells, which is seen in the aged spleen [135, 140, 141]. This impaired immune function, with possible senescence accumulation could explain the age-associated increased rate of cancer incidence in the gastrointestinal tract.

Despite the lack of direct evidence, with the support of pre-existing knowledge of age-associated functional decline and senescence accumulation in organs [191–194] and systemic vasculature [195, 196] associated with these mucosal lymphoid tissues, it is exceedingly convincing that there is an age-dependent accumulation of SnCs in these sites and/or that their functionality is somehow impacted by this accumulation. A speculative supporting argument for this is that the mucosal surfaces are exposed to more environmental stressors than most other organs, which could possibly cause low-grade chronic activation of their immune system and SnCs accumulation. This could explain why we see a relatively early onset in the aging of the mucosal immune system compared to the systemic immune system [180, 181, 186, 197].

Apart from all the circumstantial and correlative evidence, more studies are required to further our understanding of the role of cellular senescence in age-associated changes in MALT and how or if senolytics can rejuvenate them.

CONCLUSION

As summarized in (Table 1), even at an organ level, the age-associated changes that contribute to immunosenescence are multifaceted with a wide variety of undesirable phenotypic manifestations. Thus, it would be ill-advised to address each of these problems individually. A more feasible and effective way to deal with immunosenescence would be to tackle the fundamental aspects of aging that drive immunosenescence. With studies showing that clearing SnCs can rejuvenate entire tissues and organs of the aged immune system [63, 140], cellular senescence is certainly one such fundamental aspect, which has the potential to address immunosenescence.

Cellular senescence, because of its involvement in several age-related dysfunctions and disorders, has become an essential area of interest in the field of aging research. Despite a great deal of assimilated knowledge on this phenomenon, there still remain unanswered questions. The role of cellular senescence in immunosenescence is one such key area needing further exploration. With few publications addressing the direct involvement of cellular senescence in specified immunological contexts, and many more studies providing evidence for a possible role of cellular senescence in impeding the function of the immune system, this is an area of research that deserves further exploration and an investment of resources.

In this proposed pursuit, there are several “low-hanging fruit”. A few such addressable questions include: Do SnCs play a direct or indirect role in age-related disparities seen in inflammatory pathological conditions like sepsis? Does SnCs accumulation in the peripheral tissues of the body impact the functionality of immune cells in the central nervous system? Can clearing SnCs hinder the pace of thymic involution? Can clearing SnCs in combination with thymic rejuvenation therapies in the elderly improve thymic function? Does cellular senescence drive age-associated autoimmunity? Can clearing SnCs or inhibiting SASP boost the functionality of different immune cells? Does cellular senescence play a direct role in the impaired vaccination efficacy in the elderly? Is there a senostatic/senolytic regimen that can be followed before and after vaccination to boost its efficacy in the elderly?

The increasing array of genetic models of SnCs clearance along with a growing panel of senolytic and senostatic agents, provide a unique opportunity for scientists to answer these questions to lay a strong foundation to this new avenue of research in immunosenescence. Ultimately, gaining a deeper understanding of the interaction between cellular senescence and immunosenescence will help in the development of improved therapeutics that will aid in the conservation of our vitality as we age.

Table 1. Age-associated changes in the lymphoid organs that contribute to immunosenescence.

Organ	Age-Associated Changes	References
Bone Marrow	↑ Senescent Hematopoietic Stem Cells	[106, 198]
	↑ Senescent Mesenchymal Stem Cells	[95]
	↑ Adiposity	[83, 88, 99]
	↑ Myelopoiesis	[88] [78, 80, 82]
	↓ Lymphopoiesis	[88]
	↑ Oxidative Stress	[95, 96]
	↑ DNA damage	[63, 94, 95, 108, 199]
	↑ Inflammation	[95, 102]
	↓ HSC functionality	[63, 68, 77, 200]
Thymus	↓ Structural Integrity	[111, 112]
	↑ Senescent Thymic Epithelial Cells	[121]
	↑ Adipocytes	[112]
	↑ Fibrosis	[129, 201]
	↑ Inflammation	[122]
	↑ DNA damage	[121]
	↑ Oxidative Stress	[121]
	↓ Naïve T-cell turnover	[125, 126]
Spleen	↓ Structural Integrity	[135]
	↓ Macrophage Phagocytosis	[139]
	↑ Cellular Senescence	[50, 137]
	↓ Migration of B-cells	[135, 141]
	↓ Antigen Presenting Functionality	[135, 144]
	↓ Recruitment of T-cells	[143]
Lymph Nodes	↓ Number	[135, 155]
	↓ Structural Integrity	[135, 156]
	↓ Functionality	[158, 162, 202]
	↑ Adiposity	[155, 156, 158]
	↑ Fibrosis	[155, 156, 158]
Mucosa Associated Lymphoid Tissue	↓ Naïve B-cell repertoire	[186]
	↓ Naïve T-cell repertoire	[186]
	↑ Memory B-cells	[186]
	↑ Memory T-cells	[186]
	↓ Functionality	[188, 189]
	↓ Dendritic Cell Number	[190]
	↓ Dendritic Cell Functionality	[189, 190]

AUTHOR CONTRIBUTIONS

V.B. conceived and wrote the manuscript. D.Z. conceived and revised the manuscript. T.C.F. reviewed and edited the manuscript.

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CONFLICTS OF INTEREST

D.Z. is an inventor of a pending patent application for use of Bcl-xL proteolysis targeting chimeras (PROTACs) as senolytic agents, and a co-founder and stockholder of Unity Biotechnology that develops senolytics to treat age-related diseases. The other authors declare no competing interests.

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REFERENCES

1. Franceschi C, Garagnani P, Morsiani C, Conte M, Santoro A, Grignolio A, Monti D, Capri M, Salvioli S. The Continuum of Aging and Age-Related Diseases: Common Mechanisms but Different Rates. Front Med (Lausanne). 2018; 5:61. https://doi.org/10.3389/fmed.2018.00061 PMID:29662881

2. United Nations, Department of Economic and Social Affairs, Population Division (2019). World Population Ageing 2019: Highlights (ST/ESA/SER.A/430). https://www.un.org/en/development/desa/population/publications/pdf/ageing/WorldPopulationAgeing2019-Highlights.pdf.

3. Childs BG, Durik M, Baker DJ, van Deursen JM. Cellular senescence in aging and age-related disease: from mechanisms to therapy. Nat Med. 2015; 21:1424–35. https://doi.org/10.1038/nm.4000 PMID:26646499

4. Office of National Statistics. Overview of the UK population: January 2021. 2021. https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/articles/overviewoftheukpopulation/january2021.

5. Roberts AW, Bureau USC, American Community S. The population 65 years and older in the United States: 2016. https://www.census.gov/content/dam/Census/library/publications/2018/acs/ACS-38.pdf.

6. Vespa J, Armstrong DM, Medina L, Bureau USC. Demographic turning points for the United States: population projections for 2020 to 2060. https://www.census.gov/library/publications/2020/demo/p25-1144.html

7. Kontis V, Bennett JE, Mathers CD, Li G, Foreman K, Ezzati M. Future life expectancy in 35 industrialised countries: projections with a Bayesian model ensemble. Lancet. 2017; 389:1323–35. https://doi.org/10.1016/S0140-6736(16)32381-9 PMID:28236464

8. Piper MD, Bartke A. Diet and aging. Cell Metab. 2008; 8:99–104. https://doi.org/10.1016/j.cmet.2008.06.012 PMID:18680711

9. Ribarič S. Diet and aging. Oxid Med Cell Longev. 2012; 2012:741468. https://doi.org/10.1155/2012/741468 PMID:22928085

10. Garatachea N, Pareja-Galeano H, Sanchis-Gomar F, Santos-Lozano A, Fiuza-Luces C, Morán M, Emanuele E, Joyner MJ, Lucia A. Exercise attenuates the major hallmarks of aging. Rejuvenation Res. 2015; 18:57–89. https://doi.org/10.1089/rej.2014.1623 PMID:25431878

11. Schafer MJ, White TA, Evans G, Tonne JM, Verzosa GC, Stout MB, Mazula DL, Palmer AK, Baker DJ, Jensen MD, Torbenson MS, Miller JD, Ikeda Y, et al. Exercise Prevents Diet-Induced Cellular Senescence in Adipose Tissue. Diabetes. 2016; 65:1606–15. https://doi.org/10.2337/db15-0291 PMID:26983960

12. Davidsohn N, Pezone M, Vernet A, Graveline A, Oliver D, Slomovic S, Punthambaker S, Sun X, Liao R, Bonventre JV, Church GM. A single combination gene therapy treats multiple age-related diseases. Proc Natl Acad Sci U S A. 2019; 116:23505–11. https://doi.org/10.1073/pnas.1910073116 PMID:31685628

13. Qian M, Liu B. Pharmaceutical Intervention of Aging. Adv Exp Med Biol. 2018; 1086:235–54. https://doi.org/10.1007/978-981-13-1117-8_15 PMID:30232763

14. He Y, Li W, Lv D, Zhang X, Zhang X, Ortiz YT, Budamagunta V, Campisi J, Zheng G, Zhou D. Inhibition of USP7 activity selectively eliminates senescent cells in part via restoration of p53 activity. Aging Cell. 2020; 19:e13117. https://doi.org/10.1111/acel.13117 PMID:32064756

15. He Y, Zhang X, Chang J, Kim HN, Zhang P, Wang Y, Khan S, Liu X, Zhang X, Lv D, Song L, Li W, Thummuri D, et al. Using proteolysis-targeting chimera technology to reduce navitoclax platelet toxicity and improve its senolytic activity. Nat Commun. 2020; 11:1996. https://doi.org/10.1038/s41467-020-15838-0 PMID:32332723

16. Hickson LJ, Langhi Prata LGP, Bobart SA, Evans TK, Giorgadze N, Hashmi SK, Herrmann SM, Jensen MD, Jia Q, Jordan KL, Kellogg TA, Khosla S, Koerber DM, et al. Senolytics decrease senescent cells in humans: Preliminary report from a clinical trial of Dasatinib plus Quercetin in individuals with diabetic kidney disease. EBioMedicine. 2019; 47:446–56. https://doi.org/10.1016/j.ebiom.2019.08.069 PMID:31542391

17. Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. Cell. 2010; 140:619–30. https://doi.org/10.1016/j.cell.2010.02.014 PMID:20211132

18. Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, Hohmeyer A, Gereke M, Rudalska R, Potapova A, Iken M, Vucur M, Weiss S, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature. 2011; 479:547–51. https://doi.org/10.1038/nature10599 PMID:22080947

19. Yevsa T, Kang TW, Zender L. Immune surveillance of pre-cancerous senescent hepatocytes limits hepatocellular carcinoma development. Oncoimmunology. 2012; 1:398–99. https://doi.org/10.4161/onci.19128 PMID:22737629

20. Prata LGP, Ovsyannikova IG, Tchkonia T, Kirkland JL. Senescent cell clearance by the immune system: Emerging therapeutic opportunities. Semin Immunol. 2018; 40:101275. https://doi.org/10.1016/j.smim.2019.04.003 PMID:31088710

21. Song P, An J, Zou MH. Immune Clearance of Senescent Cells to Combat Ageing and Chronic Diseases. Cells. 2020; 9:671. https://doi.org/10.3390/cells9030671 PMID:32164335

22. Kale A, Sharma A, Stolzing A, Desprez PY, Campisi J. Role of immune cells in the removal of deleterious senescent cells. Immun Ageing. 2020; 17:16. https://doi.org/10.1186/s12979-020-00187-9 PMID:32518575

23. Abbas AR, Baldwin D, Ma Y, Ouyang W, Gurney A, Martin F, Fong S, van Lookeren Campagne M, Godowski P, Williams PM, Chan AC, Clark HF. Immune response in silico (IRIS): immune-specific genes identified from a compendium of microarray expression data. Genes Immun. 2005; 6:319–31. https://doi.org/10.1038/sj.gene.6364173 PMID:15789058

24. Crimmins EM. Lifespan and Healthspan: Past, Present, and Promise. Gerontologist. 2015; 55:901–11. https://doi.org/10.1093/geront/gnv130 PMID:26561272

25. Nacionales DC, Gentile LF, Vanzant E, Lopez MC, Cuenca A, Cuenca AG, Ungaro R, Li Y, Baslanti TO, Bihorac A, Moore FA, Baker HV, Leeuwenburgh C, et al. Aged mice are unable to mount an effective myeloid response to sepsis. J Immunol. 2014; 192:612–22. https://doi.org/10.4049/jimmunol.1302109 PMID:24337739

26. Brubaker AL, Rendon JL, Ramirez L, Choudhry MA, Kovacs EJ. Reduced neutrophil chemotaxis and infiltration contributes to delayed resolution of cutaneous wound infection with advanced age. J Immunol. 2013; 190:1746–57. https://doi.org/10.4049/jimmunol.1201213 PMID:23319733

27. Iuliano AD, Roguski KM, Chang HH, Muscatello DJ, Palekar R, Tempia S, Cohen C, Gran JM, Schanzer D, Cowling BJ, Wu P, Kyncl J, Ang LW, et al, and Global Seasonal Influenza-associated Mortality Collaborator Network. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. Lancet. 2018; 391:1285–300. https://doi.org/10.1016/S0140-6736(17)33293-2 PMID:29248255

28. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA. 2003; 289:179–86. https://doi.org/10.1001/jama.289.2.179 PMID:12517228

29. Demicheli V, Jefferson T, Di Pietrantonj C, Ferroni E, Thorning S, Thomas RE, Rivetti A. Vaccines for preventing influenza in the elderly. Cochrane Database Syst Rev. 2018; 2:CD004876. PMID:29388197

30. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: a quantitative review. Vaccine. 2006; 24:1159–69. https://doi.org/10.1016/j.vaccine.2005.08.105 PMID:16213065

31. Yang X, Yu Y, Xu J, Shu H, Xia J, Liu H, Wu Y, Zhang L, Yu Z, Fang M, Yu T, Wang Y, Pan S, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med. 2020; 8:475–81. https://doi.org/10.1016/S2213-2600(20)30079-5 PMID:32105632

32. Ovadya Y, Landsberger T, Leins H, Vadai E, Gal H, Biran A, Yosef R, Sagiv A, Agrawal A, Shapira A, Windheim J, Tsoory M, Schirmbeck R, et al. Impaired immune surveillance accelerates accumulation of senescent cells and aging. Nat Commun. 2018; 9:5435. https://doi.org/10.1038/s41467-018-07825-3 PMID:30575733

33. Pera A, Campos C, López N, Hassouneh F, Alonso C, Tarazona R, Solana R. Immunosenescence: Implications for response to infection and vaccination in older people. Maturitas. 2015; 82:50–55. https://doi.org/10.1016/j.maturitas.2015.05.004 PMID:26044074

34. Karin O, Agrawal A, Porat Z, Krizhanovsky V, Alon U. Senescent cell turnover slows with age providing an explanation for the Gompertz law. Nat Commun. 2019; 10:5495. https://doi.org/10.1038/s41467-019-13192-4 PMID:31792199

35. Keilich SR, Bartley JM, Haynes L. Diminished immune responses with aging predispose older adults to common and uncommon influenza complications. Cell Immunol. 2019; 345:103992. https://doi.org/10.1016/j.cellimm.2019.103992 PMID:31627841

36. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y, Xie C, Ma K, Shang K, Wang W, Tian DS. Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis. 2020; 71:762–68. https://doi.org/10.1093/cid/ciaa248 PMID:32161940

37. Goronzy JJ, Weyand CM. Immune aging and autoimmunity. Cell Mol Life Sci. 2012; 69:1615–23. PMID:22466672

38. Goronzy JJ, Shao L, Weyand CM. Immune aging and rheumatoid arthritis. Rheum Dis Clin North Am. 2010; 36:297–310. https://doi.org/10.1016/j.rdc.2010.03.001 PMID:20510235

39. Sidler C, Wóycicki R, Ilnytskyy Y, Metz G, Kovalchuk I, Kovalchuk O. Immunosenescence is associated with altered gene expression and epigenetic regulation in primary and secondary immune organs. Front Genet. 2013; 4:211. https://doi.org/10.3389/fgene.2013.00211 PMID:24151501

40. Turner JE. Is immunosenescence influenced by our lifetime "dose" of exercise? Biogerontology. 2016; 17:581–602. PMID:27023222

41. Maijó M, Clements SJ, Ivory K, Nicoletti C, Carding SR. Nutrition, diet and immunosenescence. Mech Ageing Dev. 2014; 136–137:116–28. https://doi.org/10.1016/j.mad.2013.12.003 PMID:24373813

42. Caruso C, Accardi G, Virruso C, Candore G. Sex, gender and immunosenescence: a key to understand the different lifespan between men and women? Immun Ageing. 2013; 10:20. https://doi.org/10.1186/1742-4933-10-20 PMID:23680476

43. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res. 1961; 25:585–621. https://doi.org/10.1016/0014-4827(61)90192-6 PMID:13905658

44. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007; 8:729–40. https://doi.org/10.1038/nrm2233 PMID:17667954

45. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. Cell. 2007; 130:223–33. https://doi.org/10.1016/j.cell.2007.07.003 PMID:17662938

46. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L, Lowe SW. Senescence of activated stellate cells limits liver fibrosis. Cell. 2008; 134:657–67. https://doi.org/10.1016/j.cell.2008.06.049 PMID:18724938

47. Kim KH, Chen CC, Monzon RI, Lau LF. Matricellular protein CCN1 promotes regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblasts. Mol Cell Biol. 2013; 33:2078–90. https://doi.org/10.1128/MCB.00049-13 PMID:23508104

48. Cui S, Xue L, Yang F, Dai S, Han Z, Liu K, Liu B, Yuan Q, Cui Z, Zhang Y, Xu F, Chen Y. Postinfarction Hearts Are Protected by Premature Senescent Cardiomyocytes Via GATA 4-Dependent CCN 1 Secretion. J Am Heart Assoc. 2018; 7:e009111. https://doi.org/10.1161/JAHA.118.009111 PMID:30371213

49. Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, Laberge RM, Vijg J, Van Steeg H, Dollé ME, Hoeijmakers JH, de Bruin A, Hara E, Campisi J. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev Cell. 2014; 31:722–33. https://doi.org/10.1016/j.devcel.2014.11.012 PMID:25499914

50. Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, Saltness RA, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaie K, Miller JD, van Deursen JM. Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. Nature. 2016; 530:184–89. https://doi.org/10.1038/nature16932 PMID:26840489

51. Coppé JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol. 2010; 5:99–118. https://doi.org/10.1146/annurev-pathol-121808-102144 PMID:20078217

52. Lasry A, Ben-Neriah Y. Senescence-associated inflammatory responses: aging and cancer perspectives. Trends Immunol. 2015; 36:217–28. https://doi.org/10.1016/j.it.2015.02.009 PMID:25801910

53. He S, Sharpless NE. Senescence in Health and Disease. Cell. 2017; 169:1000–11. https://doi.org/10.1016/j.cell.2017.05.015 PMID:28575665

54. Lewis-McDougall FC, Ruchaya PJ, Domenjo-Vila E, Shin Teoh T, Prata L, Cottle BJ, Clark JE, Punjabi PP, Awad W, Torella D, Tchkonia T, Kirkland JL, Ellison-Hughes GM. Aged-senescent cells contribute to impaired heart regeneration. Aging Cell. 2019; 18:e12931. https://doi.org/10.1111/acel.12931 PMID:30854802

55. Ogrodnik M, Zhu Y, Langhi LGP, Tchkonia T, Krüger P, Fielder E, Victorelli S, Ruswhandi RA, Giorgadze N, Pirtskhalava T, Podgorni O, Enikolopov G, Johnson KO, et al. Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. Cell Metab. 2019; 29:1233. https://doi.org/10.1016/j.cmet.2019.01.013 PMID:31067450

56. Minamino T, Miyauchi H, Yoshida T, Ishida Y, Yoshida H, Komuro I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. Circulation. 2002; 105:1541–44. https://doi.org/10.1161/01.cir.0000013836.85741.17 PMID:11927518

57. Chimenti C, Kajstura J, Torella D, Urbanek K, Heleniak H, Colussi C, Di Meglio F, Nadal-Ginard B, Frustaci A, Leri A, Maseri A, Anversa P. Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure. Circ Res. 2003; 93:604–13. https://doi.org/10.1161/01.RES.0000093985.76901.AF PMID:12958145

58. Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM, Inman CL, Ogrodnik MB, Hachfeld CM, Fraser DG, Onken JL, Johnson KO, Verzosa GC, et al. Senolytics improve physical function and increase lifespan in old age. Nat Med. 2018; 24:1246–56. https://doi.org/10.1038/s41591-018-0092-9 PMID:29988130

59. Sun R, Zhu B, Xiong K, Sun Y, Shi D, Chen L, Zhang Y, Li Z, Xue L. Senescence as a novel mechanism involved in β-adrenergic receptor mediated cardiac hypertrophy. PLoS One. 2017; 12:e0182668. https://doi.org/10.1371/journal.pone.0182668 PMID:28783759

60. Nelson G, Wordsworth J, Wang C, Jurk D, Lawless C, Martin-Ruiz C, von Zglinicki T. A senescent cell bystander effect: senescence-induced senescence. Aging Cell. 2012; 11:345–49. https://doi.org/10.1111/j.1474-9726.2012.00795.x PMID:22321662

61. da Silva PFL, Ogrodnik M, Kucheryavenko O, Glibert J, Miwa S, Cameron K, Ishaq A, Saretzki G, Nagaraja-Grellscheid S, Nelson G, von Zglinicki T. The bystander effect contributes to the accumulation of senescent cells *in vivo*. Aging Cell. 2019; 18:e12848. https://doi.org/10.1111/acel.12848 PMID:30462359

62. Peng X, Wu Y, Brouwer U, van Vliet T, Wang B, Demaria M, Barazzuol L, Coppes RP. Cellular senescence contributes to radiation-induced hyposalivation by affecting the stem/progenitor cell niche. Cell Death Dis. 2020; 11:854. https://doi.org/10.1038/s41419-020-03074-9 PMID:33056980

63. Chang J, Wang Y, Shao L, Laberge RM, Demaria M, Campisi J, Janakiraman K, Sharpless NE, Ding S, Feng W, Luo Y, Wang X, Aykin-Burns N, et al. Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. Nat Med. 2016; 22:78–83. https://doi.org/10.1038/nm.4010 PMID:26657143

64. Sagiv A, Burton DG, Moshayev Z, Vadai E, Wensveen F, Ben-Dor S, Golani O, Polic B, Krizhanovsky V. NKG2D ligands mediate immunosurveillance of senescent cells. Aging (Albany NY). 2016; 8:328–44. https://doi.org/10.18632/aging.100897 PMID:26878797

65. Fuentes E, Fuentes M, Alarcón M, Palomo I. Immune System Dysfunction in the Elderly. An Acad Bras Cienc. 2017; 89:285–99. https://doi.org/10.1590/0001-3765201720160487 PMID:28423084

66. Pereira BI, Devine OP, Vukmanovic-Stejic M, Chambers ES, Subramanian P, Patel N, Virasami A, Sebire NJ, Kinsler V, Valdovinos A, LeSaux CJ, Passos JF, Antoniou A, et al. Senescent cells evade immune clearance via HLA-E-mediated NK and $CD8^+$ T cell inhibition. Nat Commun. 2019; 10:2387. https://doi.org/10.1038/s41467-019-10335-5 PMID:31160572

67. Muñoz DP, Yannone SM, Daemen A, Sun Y, Vakar-Lopez F, Kawahara M, Freund AM, Rodier F, Wu JD, Desprez PY, Raulet DH, Nelson PS, van 't Veer LJ, et al. Targetable mechanisms driving immunoevasion of persistent senescent cells link chemotherapy-resistant cancer to aging. JCI Insight. 2019; 5:e124716.

https://doi.org/10.1172/jci.insight.124716
PMID:31184599

68. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature. 2006; 443:421–26.
https://doi.org/10.1038/nature05159
PMID:16957735

69. Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ. Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature. 2006; 443:448–52.
https://doi.org/10.1038/nature05091
PMID:16957738

70. Josephson AM, Bradaschia-Correa V, Lee S, Leclerc K, Patel KS, Muinos Lopez E, Litwa HP, Neibart SS, Kadiyala M, Wong MZ, Mizrahi MM, Yim NL, Ramme AJ, et al. Age-related inflammation triggers skeletal stem/progenitor cell dysfunction. Proc Natl Acad Sci U S A. 2019; 116:6995–7004.
https://doi.org/10.1073/pnas.1810692116
PMID:30894483

71. Zhao J, Zhang L, Lu A, Han Y, Colangelo D, Bukata C, Scibetta A, Yousefzadeh MJ, Li X, Gurkar AU, McGowan SJ, Angelini L, O'Kelly R, et al. ATM is a key driver of NF-κB-dependent DNA-damage-induced senescence, stem cell dysfunction and aging. Aging (Albany NY). 2020; 12:4688–710.
https://doi.org/10.18632/aging.102863
PMID:32201398

72. Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE. p16INK4a induces an age-dependent decline in islet regenerative potential. Nature. 2006; 443:453–57.
https://doi.org/10.1038/nature05092
PMID:16957737

73. Zhao E, Xu H, Wang L, Kryczek I, Wu K, Hu Y, Wang G, Zou W. Bone marrow and the control of immunity. Cell Mol Immunol. 2012; 9:11–19.
https://doi.org/10.1038/cmi.2011.47
PMID:22020068

74. Muschler GF, Nitto H, Boehm CA, Easley KA. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. J Orthop Res. 2001; 19:117–25.
https://doi.org/10.1016/S0736-0266(00)00010-3
PMID:11332607

75. Pritz T, Weinberger B, Grubeck-Loebenstein B. The aging bone marrow and its impact on immune responses in old age. Immunol Lett. 2014; 162:310–15.
https://doi.org/10.1016/j.imlet.2014.06.016
PMID:25014741

76. Hoffman CM, Han J, Calvi LM. Impact of aging on bone, marrow and their interactions. Bone. 2019; 119:1–7.
https://doi.org/10.1016/j.bone.2018.07.012
PMID:30010082

77. Rossi DJ, Bryder D, Zahn JM, Ahlenius H, Sonu R, Wagers AJ, Weissman IL. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A. 2005; 102:9194–99.
https://doi.org/10.1073/pnas.0503280102
PMID:15967997

78. Pang WW, Price EA, Sahoo D, Beerman I, Maloney WJ, Rossi DJ, Schrier SL, Weissman IL. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci U S A. 2011; 108:20012–17.
https://doi.org/10.1073/pnas.1116110108
PMID:22123971

79. Ogawa T, Kitagawa M, Hirokawa K. Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages. Mech Ageing Dev. 2000; 117:57–68.
https://doi.org/10.1016/s0047-6374(00)00137-8
PMID:10958923

80. Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood. 2005; 106:1479–87.
https://doi.org/10.1182/blood-2004-11-4282
PMID:15827136

81. Vas V, Senger K, Dörr K, Niebel A, Geiger H. Aging of the microenvironment influences clonality in hematopoiesis. PLoS One. 2012; 7:e42080.
https://doi.org/10.1371/journal.pone.0042080
PMID:22879906

82. Ergen AV, Boles NC, Goodell MA. Rantes/Ccl5 influences hematopoietic stem cell subtypes and causes myeloid skewing. Blood. 2012; 119:2500–09.
https://doi.org/10.1182/blood-2011-11-391730
PMID:22289892

83. Takeshita S, Fumoto T, Naoe Y, Ikeda K. Age-related marrow adipogenesis is linked to increased expression of RANKL. J Biol Chem. 2014; 289:16699–710.
https://doi.org/10.1074/jbc.M114.547919
PMID:24753250

84. Umemoto Y, Tsuji K, Yang FC, Ebihara Y, Kaneko A, Furukawa S, Nakahata T. Leptin stimulates the proliferation of murine myelocytic and primitive hematopoietic progenitor cells. Blood. 1997; 90:3438–43.
PMID:9345027

85. Shimozato T, Kincade PW. Prostaglandin E(2) and stem cell factor can deliver opposing signals to B lymphocyte precursors. Cell Immunol. 1999; 198:21–29. https://doi.org/10.1006/cimm.1999.1575 PMID:10612648

86. Yokota T, Meka CS, Kouro T, Medina KL, Igarashi H, Takahashi M, Oritani K, Funahashi T, Tomiyama Y, Matsuzawa Y, Kincade PW. Adiponectin, a fat cell product, influences the earliest lymphocyte precursors in bone marrow cultures by activation of the cyclooxygenase-prostaglandin pathway in stromal cells. J Immunol. 2003; 171:5091–99. https://doi.org/10.4049/jimmunol.171.10.5091 PMID:14607907

87. Poloni A, Maurizi G, Serrani F, Mancini S, Zingaretti MC, Frontini A, Cinti S, Olivieri A, Leoni P. Molecular and functional characterization of human bone marrow adipocytes. Exp Hematol. 2013; 41:558–66.e2. https://doi.org/10.1016/j.exphem.2013.02.005 PMID:23435314

88. Wang H, Leng Y, Gong Y. Bone Marrow Fat and Hematopoiesis. Front Endocrinol (Lausanne). 2018; 9:694. https://doi.org/10.3389/fendo.2018.00694 PMID:30546345

89. Farr JN, Fraser DG, Wang H, Jaehn K, Ogrodnik MB, Weivoda MM, Drake MT, Tchkonia T, LeBrasseur NK, Kirkland JL, Bonewald LF, Pignolo RJ, Monroe DG, Khosla S. Identification of Senescent Cells in the Bone Microenvironment. J Bone Miner Res. 2016; 31:1920–29. https://doi.org/10.1002/jbmr.2892 PMID:27341653

90. Liu J, Ding Y, Liu Z, Liang X. Senescence in Mesenchymal Stem Cells: Functional Alterations, Molecular Mechanisms, and Rejuvenation Strategies. Front Cell Dev Biol. 2020; 8:258. https://doi.org/10.3389/fcell.2020.00258 PMID:32478063

91. Kim HN, Xiong J, MacLeod RS, Iyer S, Fujiwara Y, Cawley KM, Han L, He Y, Thostenson JD, Ferreira E, Jilka RL, Zhou D, Almeida M, O'Brien CA. Osteocyte RANKL is required for cortical bone loss with age and is induced by senescence. JCI Insight. 2020; 5:e138815. https://doi.org/10.1172/jci.insight.138815 PMID:32870816

92. Eyman D, Damodarasamy M, Plymate SR, Reed MJ. CCL5 secreted by senescent aged fibroblasts induces proliferation of prostate epithelial cells and expression of genes that modulate angiogenesis. J Cell Physiol. 2009; 220:376–81. https://doi.org/10.1002/jcp.21776 PMID:19360811

93. Liu ZJ, Tan Y, Beecham GW, Seo DM, Tian R, Li Y, Vazquez-Padron RI, Pericak-Vance M, Vance JM, Goldschmidt-Clermont PJ, Livingstone AS, Velazquez OC. Notch activation induces endothelial cell senescence and pro-inflammatory response: implication of Notch signaling in atherosclerosis. Atherosclerosis. 2012; 225:296–303. https://doi.org/10.1016/j.atherosclerosis.2012.04.010 PMID:23078884

94. Minieri V, Saviozzi S, Gambarotta G, Lo Iacono M, Accomasso L, Cibrario Rocchietti E, Gallina C, Turinetto V, Giachino C. Persistent DNA damage-induced premature senescence alters the functional features of human bone marrow mesenchymal stem cells. J Cell Mol Med. 2015; 19:734–43. https://doi.org/10.1111/jcmm.12387 PMID:25619736

95. Gnani D, Crippa S, Della Volpe L, Rossella V, Conti A, Lettera E, Rivis S, Ometti M, Fraschini G, Bernardo ME, Di Micco R. An early-senescence state in aged mesenchymal stromal cells contributes to hematopoietic stem and progenitor cell clonogenic impairment through the activation of a pro-inflammatory program. Aging Cell. 2019; 18:e12933. https://doi.org/10.1111/acel.12933 PMID:30828977

96. Khatri R, Krishnan S, Roy S, Chattopadhyay S, Kumar V, Mukhopadhyay A. Reactive Oxygen Species Limit the Ability of Bone Marrow Stromal Cells to Support Hematopoietic Reconstitution in Aging Mice. Stem Cells Dev. 2016; 25:948–58. https://doi.org/10.1089/scd.2015.0391 PMID:27140293

97. Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, Khosla S, Jensen MD, Kirkland JL. Fat tissue, aging, and cellular senescence. Aging Cell. 2010; 9:667–84. https://doi.org/10.1111/j.1474-9726.2010.00608.x PMID:20701600

98. Farr JN, Xu M, Weivoda MM, Monroe DG, Fraser DG, Onken JL, Negley BA, Sfeir JG, Ogrodnik MB, Hachfeld CM, LeBrasseur NK, Drake MT, Pignolo RJ, et al. Corrigendum: Targeting cellular senescence prevents age-related bone loss in mice. Nat Med. 2017; 23:1384. https://doi.org/10.1038/nm1117-1384c PMID:29117174

99. Ambrosi TH, Scialdone A, Graja A, Gohlke S, Jank AM, Bocian C, Woelk L, Fan H, Logan DW, Schürmann A, Saraiva LR, Schulz TJ. Adipocyte Accumulation in the Bone Marrow during Obesity and Aging Impairs Stem Cell-Based Hematopoietic and Bone Regeneration. Cell Stem Cell. 2017; 20:771–84.e6.

https://doi.org/10.1016/j.stem.2017.02.009
PMID:28330582

100. Naveiras O, Nardi V, Wenzel PL, Hauschka PV, Fahey F, Daley GQ. Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. Nature. 2009; 460:259–63. https://doi.org/10.1038/nature08099 PMID:19516257

101. Stephan RP, Reilly CR, Witte PL. Impaired ability of bone marrow stromal cells to support B-lymphopoiesis with age. Blood. 1998; 91:75–88. PMID:9414271

102. Ueda Y, Yang K, Foster SJ, Kondo M, Kelsoe G. Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. J Exp Med. 2004; 199:47–58. https://doi.org/10.1084/jem.20031104 PMID:14707114

103. Hagen M, Derudder E. Inflammation and the Alteration of B-Cell Physiology in Aging. Gerontology. 2020; 66:105–13. https://doi.org/10.1159/000501963 PMID:31553969

104. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Ryan DH. TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. J Immunol. 1997; 159:117–25. PMID:9200446

105. Day RB, Bhattacharya D, Nagasawa T, Link DC. Granulocyte colony-stimulating factor reprograms bone marrow stromal cells to actively suppress B lymphopoiesis in mice. Blood. 2015; 125:3114–17. https://doi.org/10.1182/blood-2015-02-629444 PMID:25814527

106. Fali T, Fabre-Mersseman V, Yamamoto T, Bayard C, Papagno L, Fastenackels S, Zoorab R, Koup RA, Boddaert J, Sauce D, Appay V. Elderly human hematopoietic progenitor cells express cellular senescence markers and are more susceptible to pyroptosis. JCI Insight. 2018; 3:e95319. https://doi.org/10.1172/jci.insight.95319 PMID:29997288

107. Nelson G, Kucheryavenko O, Wordsworth J, von Zglinicki T. The senescent bystander effect is caused by ROS-activated NF-κB signalling. Mech Ageing Dev. 2018; 170:30–36. https://doi.org/10.1016/j.mad.2017.08.005 PMID:28837845

108. Beerman I, Seita J, Inlay MA, Weissman IL, Rossi DJ. Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. Cell Stem Cell. 2014; 15:37–50. https://doi.org/10.1016/j.stem.2014.04.016 PMID:24813857

109. Mohrin M, Bourke E, Alexander D, Warr MR, Barry-Holson K, Le Beau MM, Morrison CG, Passegué E. Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell. 2010; 7:174–85. https://doi.org/10.1016/j.stem.2010.06.014 PMID:20619762

110. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature. 2007; 447:725–29. https://doi.org/10.1038/nature05862 PMID:17554309

111. Aw D, Silva AB, Maddick M, von Zglinicki T, Palmer DB. Architectural changes in the thymus of aging mice. Aging Cell. 2008; 7:158–67. https://doi.org/10.1111/j.1474-9726.2007.00365.x PMID:18241323

112. Cavallotti C, D'Andrea V, Tonnarini G, Cavallotti C, Bruzzone P. Age-related changes in the human thymus studied with scanning electron microscopy. Microsc Res Tech. 2008; 71:573–78. https://doi.org/10.1002/jemt.20588 PMID:18398833

113. Varecza Z, Kvell K, Talabér G, Miskei G, Csongei V, Bartis D, Anderson G, Jenkinson EJ, Pongracz JE. Multiple suppression pathways of canonical Wnt signalling control thymic epithelial senescence. Mech Ageing Dev. 2011; 132:249–56. https://doi.org/10.1016/j.mad.2011.04.007 PMID:21549744

114. Kvell K, Varecza Z, Bartis D, Hesse S, Parnell S, Anderson G, Jenkinson EJ, Pongracz JE. Wnt4 and LAP2alpha as pacemakers of thymic epithelial senescence. PLoS One. 2010; 5:e10701. https://doi.org/10.1371/journal.pone.0010701 PMID:20502698

115. Aw D, Taylor-Brown F, Cooper K, Palmer DB. Phenotypical and morphological changes in the thymic microenvironment from ageing mice. Biogerontology. 2009; 10:311–22. https://doi.org/10.1007/s10522-008-9182-2 PMID:18931936

116. Shanley DP, Aw D, Manley NR, Palmer DB. An evolutionary perspective on the mechanisms of immunosenescence. Trends Immunol. 2009; 30:374–81. https://doi.org/10.1016/j.it.2009.05.001 PMID:19541538

117. Petrie HT. Role of thymic organ structure and stromal composition in steady-state postnatal T-cell production. Immunol Rev. 2002; 189:8–19. https://doi.org/10.1034/j.1600-065x.2002.18902.x PMID:12445261

118. Goronzy JJ, Lee WW, Weyand CM. Aging and T-cell diversity. Exp Gerontol. 2007; 42:400–06. https://doi.org/10.1016/j.exger.2006.11.016 PMID:17218073

119. Coder BD, Wang H, Ruan L, Su DM. Thymic involution perturbs negative selection leading to autoreactive T cells that induce chronic inflammation. J Immunol. 2015; 194:5825–37. https://doi.org/10.4049/jimmunol.1500082 PMID:25957168

120. Thomas R, Wang W, Su DM. Contributions of Age-Related Thymic Involution to Immunosenescence and Inflammaging. Immun Ageing. 2020; 17:2. https://doi.org/10.1186/s12979-020-0173-8 PMID:31988649

121. Barbouti A, Evangelou K, Pateras IS, Papoudou-Bai A, Patereli A, Stefanaki K, Rontogianni D, Muñoz-Espín D, Kanavaros P, Gorgoulis VG. *In situ* evidence of cellular senescence in Thymic Epithelial Cells (TECs) during human thymic involution. Mech Ageing Dev. 2019; 177:88–90. https://doi.org/10.1016/j.mad.2018.02.005 PMID:29490231

122. Sempowski GD, Hale LP, Sundy JS, Massey JM, Koup RA, Douek DC, Patel DD, Haynes BF. Leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA expression in human thymus increases with age and is associated with thymic atrophy. J Immunol. 2000; 164:2180–87. https://doi.org/10.4049/jimmunol.164.4.2180 PMID:10657672

123. Griffith AV, Venables T, Shi J, Farr A, van Remmen H, Szweda L, Fallahi M, Rabinovitch P, Petrie HT. Metabolic Damage and Premature Thymus Aging Caused by Stromal Catalase Deficiency. Cell Rep. 2015; 12:1071–79. https://doi.org/10.1016/j.celrep.2015.07.008 PMID:26257169

124. Takaba H, Takayanagi H. The Mechanisms of T Cell Selection in the Thymus. Trends Immunol. 2017; 38:805–16. https://doi.org/10.1016/j.it.2017.07.010 PMID:28830733

125. Gui J, Zhu X, Dohkan J, Cheng L, Barnes PF, Su DM. The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. Int Immunol. 2007; 19:1201–11. https://doi.org/10.1093/intimm/dxm095 PMID:17804689

126. Zhu X, Gui J, Dohkan J, Cheng L, Barnes PF, Su DM. Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution. Aging Cell. 2007; 6:663–72. https://doi.org/10.1111/j.1474-9726.2007.00325.x PMID:17681038

127. Becklund BR, Purton JF, Ramsey C, Favre S, Vogt TK, Martin CE, Spasova DS, Sarkisyan G, LeRoy E, Tan JT, Wahlus H, Bondi-Boyd B, Luther SA, Surh CD. The aged lymphoid tissue environment fails to support naïve T cell homeostasis. Sci Rep. 2016; 6:30842. https://doi.org/10.1038/srep30842 PMID:27480406

128. Mackall CL, Punt JA, Morgan P, Farr AG, Gress RE. Thymic function in young/old chimeras: substantial thymic T cell regenerative capacity despite irreversible age-associated thymic involution. Eur J Immunol. 1998; 28:1886–93. https://doi.org/10.1002/(SICI)1521-4141(199806)28:06<1886::AID-IMMU1886>3.0.CO;2-M PMID:9645370

129. Thompson HL, Smithey MJ, Uhrlaub JL, Jeftić I, Jergović M, White SE, Currier N, Lang AM, Okoye A, Park B, Picker LJ, Surh CD, Nikolich-Žugich J. Lymph nodes as barriers to T-cell rejuvenation in aging mice and nonhuman primates. Aging Cell. 2019; 18:e12865. https://doi.org/10.1111/acel.12865 PMID:30430748

130. Fontana L, Nehme J, Demaria M. Caloric restriction and cellular senescence. Mech Ageing Dev. 2018; 176:19–23. https://doi.org/10.1016/j.mad.2018.10.005 PMID:30395873

131. Longo VD, Cortellino S. Fasting, dietary restriction, and immunosenescence. J Allergy Clin Immunol. 2020; 146:1002–04. https://doi.org/10.1016/j.jaci.2020.07.035 PMID:32853639

132. Chung HY, Kim DH, Bang E, Yu BP. Impacts of Calorie Restriction and Intermittent Fasting on Health and Diseases: Current Trends. Nutrients. 2020; 12:2948. https://doi.org/10.3390/nu12102948 PMID:32992924

133. Yang H, Youm YH, Dixit VD. Inhibition of thymic adipogenesis by caloric restriction is coupled with reduction in age-related thymic involution. J Immunol. 2009; 183:3040–52. https://doi.org/10.4049/jimmunol.0900562 PMID:19648267

134. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. Immunity. 2013; 39:806–18. https://doi.org/10.1016/j.immuni.2013.10.010 PMID:24238338

135. Turner VM, Mabbott NA. Influence of ageing on the microarchitecture of the spleen and lymph nodes. Biogerontology. 2017; 18:723–38. https://doi.org/10.1007/s10522-017-9707-7 PMID:28501894

136. Aw D, Hilliard L, Nishikawa Y, Cadman ET, Lawrence RA, Palmer DB. Disorganization of the splenic microanatomy in ageing mice. Immunology. 2016; 148:92–101. https://doi.org/10.1111/imm.12590 PMID:26840375

137. Wang C, Jurk D, Maddick M, Nelson G, Martin-Ruiz C, von Zglinicki T. DNA damage response and cellular senescence in tissues of aging mice. Aging Cell. 2009; 8:311–23. https://doi.org/10.1111/j.1474-9726.2009.00481.x PMID:19627270

138. Park J, Miyakawa T, Shiokawa A, Nakajima-Adachi H, Tanokura M, Hachimura S. Splenic stromal cells from aged mice produce higher levels of IL-6 compared to young mice. Mediators Inflamm. 2014; 2014:826987. https://doi.org/10.1155/2014/826987 PMID:24729663

139. Birjandi SZ, Ippolito JA, Ramadorai AK, Witte PL. Alterations in marginal zone macrophages and marginal zone B cells in old mice. J Immunol. 2011; 186:3441–51. https://doi.org/10.4049/jimmunol.1001271 PMID:21307289

140. Palacio L, Goyer ML, Maggiorani D, Espinosa A, Villeneuve N, Bourbonnais S, Moquin-Beaudry G, Le O, Demaria M, Davalos AR, Decaluwe H, Beauséjour C. Restored immune cell functions upon clearance of senescence in the irradiated splenic environment. Aging Cell. 2019; 18:e12971. https://doi.org/10.1111/acel.12971 PMID:31148373

141. Turner VM, Mabbott NA. Ageing adversely affects the migration and function of marginal zone B cells. Immunology. 2017; 151:349–62. https://doi.org/10.1111/imm.12737 PMID:28369800

142. Turner DL, Bickham KL, Farber DL, Lefrançois L. Splenic priming of virus-specific CD8 T cells following influenza virus infection. J Virol. 2013; 87:4496–506. https://doi.org/10.1128/JVI.03413-12 PMID:23388712

143. Lefebvre JS, Maue AC, Eaton SM, Lanthier PA, Tighe M, Haynes L. The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. Aging Cell. 2012; 11:732–40. https://doi.org/10.1111/j.1474-9726.2012.00836.x PMID:22607653

144. Li G, Smithey MJ, Rudd BD, Nikolich-Žugich J. Age-associated alterations in CD8α+ dendritic cells impair CD8 T-cell expansion in response to an intracellular bacterium. Aging Cell. 2012; 11:968–77. https://doi.org/10.1111/j.1474-9726.2012.00867.x PMID:22862959

145. Zhu Y, Tchkonia T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, Palmer AK, Ikeno Y, Hubbard GB, Lenburg M, O'Hara SP, LaRusso NF, Miller JD, et al. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell. 2015; 14:644–58. https://doi.org/10.1111/acel.12344 PMID:25754370

146. Zhu Y, Doornebal EJ, Pirtskhalava T, Giorgadze N, Wentworth M, Fuhrmann-Stroissnigg H, Niedernhofer LJ, Robbins PD, Tchkonia T, Kirkland JL. New agents that target senescent cells: the flavone, fisetin, and the BCL-X_L inhibitors, A1331852 and A1155463. Aging (Albany NY). 2017; 9:955–63. https://doi.org/10.18632/aging.101202 PMID:28273655

147. Martin N, Soriani O, Bernard D. Cardiac Glycosides as Senolytic Compounds. Trends Mol Med. 2020; 26:243–45. https://doi.org/10.1016/j.molmed.2020.01.001 PMID:31983612

148. González-Gualda E, Pàez-Ribes M, Lozano-Torres B, Macias D, Wilson JR 3rd, González-López C, Ou HL, Mirón-Barroso S, Zhang Z, Lérida-Viso A, Blandez JF, Bernardos A, Sancenón F, et al. Galacto-conjugation of Navitoclax as an efficient strategy to increase senolytic specificity and reduce platelet toxicity. Aging Cell. 2020; 19:e13142. https://doi.org/10.1111/acel.13142 PMID:32233024

149. Kang C. Senolytics and Senostatics: A Two-Pronged Approach to Target Cellular Senescence for Delaying Aging and Age-Related Diseases. Mol Cells. 2019; 42:821–27. https://doi.org/10.14348/molcells.2019.0298 PMID:31838837

150. Willard-Mack CL. Normal structure, function, and histology of lymph nodes. Toxicol Pathol. 2006; 34:409–24. https://doi.org/10.1080/01926230600867727 PMID:17067937

151. Bousso P. T-cell activation by dendritic cells in the lymph node: lessons from the movies. Nat Rev Immunol. 2008; 8:675–84. https://doi.org/10.1038/nri2379 PMID:19172690

152. Capece T, Kim M. The Role of Lymphatic Niches in T Cell Differentiation. Mol Cells. 2016; 39:515–23. https://doi.org/10.14348/molcells.2016.0089 PMID:27306645

153. Woodland DL, Kohlmeier JE. Migration, maintenance and recall of memory T cells in peripheral tissues. Nat Rev Immunol. 2009; 9:153–61. https://doi.org/10.1038/nri2496 PMID:19240755

154. Liao S, von der Weid PY. Lymphatic system: an active pathway for immune protection. Semin Cell Dev Biol. 2015; 38:83–89. https://doi.org/10.1016/j.semcdb.2014.11.012 PMID:25534659

155. Ahmadi O, McCall JL, Stringer MD. Does senescence affect lymph node number and morphology? A systematic review. ANZ J Surg. 2013; 83:612–18. https://doi.org/10.1111/ans.12067 PMID:23347421

156. Hadamitzky C, Spohr H, Debertin AS, Guddat S, Tsokos M, Pabst R. Age-dependent histoarchitectural changes in human lymph nodes: an underestimated process with clinical relevance? J Anat. 2010; 216:556–62. https://doi.org/10.1111/j.1469-7580.2010.01213.x PMID:20345860

157. Richner JM, Gmyrek GB, Govero J, Tu Y, van der Windt GJ, Metcalf TU, Haddad EK, Textor J, Miller MJ, Diamond MS. Age-Dependent Cell Trafficking Defects in Draining Lymph Nodes Impair Adaptive Immunity and Control of West Nile Virus Infection. PLoS Pathog. 2015; 11:e1005027. https://doi.org/10.1371/journal.ppat.1005027 PMID:26204259

158. Thompson HL, Smithey MJ, Surh CD, Nikolich-Žugich J. Functional and Homeostatic Impact of Age-Related Changes in Lymph Node Stroma. Front Immunol. 2017; 8:706. https://doi.org/10.3389/fimmu.2017.00706 PMID:28659930

159. Zhao J, Zhao J, Legge K, Perlman S. Age-related increases in PGD(2) expression impair respiratory DC migration, resulting in diminished T cell responses upon respiratory virus infection in mice. J Clin Invest. 2011; 121:4921–30. https://doi.org/10.1172/JCI59777 PMID:22105170

160. Basisty N, Kale A, Jeon OH, Kuehnemann C, Payne T, Rao C, Holtz A, Shah S, Sharma V, Ferrucci L, Campisi J, Schilling B. A proteomic atlas of senescence-associated secretomes for aging biomarker development. PLoS Biol. 2020; 18:e3000599. https://doi.org/10.1371/journal.pbio.3000599 PMID:31945054

161. Masters AR, Hall A, Bartley JM, Keilich SR, Lorenzo EC, Jellison ER, Puddington L, Haynes L. Assessment of Lymph Node Stromal Cells as an Underlying Factor in Age-Related Immune Impairment. J Gerontol A Biol Sci Med Sci. 2019; 74:1734–43. https://doi.org/10.1093/gerona/glz029 PMID:30721932

162. Denton AE, Silva-Cayetano A, Dooley J, Hill DL, Carr EJ, Robert PA, Meyer-Hermann M, Liston A, Linterman MA. Intrinsic defects in lymph node stromal cells underpin poor germinal center responses during aging. bioRxiv. 2020. https://doi.org/10.1101/2020.05.07.082255

163. Dasoveanu DC, Park HJ, Ly CL, Shipman WD, Chyou S, Kumar V, Tarlinton D, Ludewig B, Mehrara BJ, Lu TT. Lymph node stromal CCL2 limits antibody responses. Sci Immunol. 2020; 5:eaaw0693. https://doi.org/10.1126/sciimmunol.aaw0693 PMID:32198221

164. Randall TD. Bronchus-associated lymphoid tissue (BALT) structure and function. Adv Immunol. 2010; 107:187–241. https://doi.org/10.1016/B978-0-12-381300-8.00007-1 PMID:21034975

165. Hwang JY, Randall TD, Silva-Sanchez A. Inducible Bronchus-Associated Lymphoid Tissue: Taming Inflammation in the Lung. Front Immunol. 2016; 7:258. https://doi.org/10.3389/fimmu.2016.00258 PMID:27446088

166. Knop N, Knop E. Conjunctiva-associated lymphoid tissue in the human eye. Invest Ophthalmol Vis Sci. 2000; 41:1270–79. PMID:10798640

167. Mastropasqua R, Agnifili L, Fasanella V, Nubile M, Gnama AA, Falconio G, Perri P, Di Staso S, Mariotti C. The Conjunctiva-Associated Lymphoid Tissue in Chronic Ocular Surface Diseases. Microsc Microanal. 2017; 23:697–707. https://doi.org/10.1017/S1431927617000538 PMID:28480834

168. Kracke A, Hiller AS, Tschernig T, Kasper M, Kleemann WJ, Tröger HD, Pabst R. Larynx-associated lymphoid tissue (LALT) in young children. Anat Rec. 1997; 248:413–20.

https://doi.org/10.1002/(SICI)1097-0185(199707)248:3<413::AID-AR14>3.0.CO;2-S PMID:9214559

169. Ono S, Kabashima K. Novel insights into the role of immune cells in skin and inducible skin-associated lymphoid tissue (iSALT). Allergo J Int. 2015; 24:170–79. https://doi.org/10.1007/s40629-015-0065-1 PMID:27069837

170. Ono S, Kabashima K. Proposal of inducible skin-associated lymphoid tissue (iSALT). Exp Dermatol. 2015; 24:630–31. https://doi.org/10.1111/exd.12716 PMID:25865042

171. Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM, van Breda Vriesman PJ, Sminia T. The role of nasopharyngeal lymphoid tissue. Immunol Today. 1992; 13:219–24. https://doi.org/10.1016/0167-5699(92)90158-4 PMID:1627250

172. Debertin AS, Tschernig T, Tönjes H, Kleemann WJ, Tröger HD, Pabst R. Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. Clin Exp Immunol. 2003; 134:503–07. https://doi.org/10.1111/j.1365-2249.2003.02311.x PMID:14632758

173. Jung C, Hugot JP, Barreau F. Peyer's Patches: The Immune Sensors of the Intestine. Int J Inflam. 2010; 2010:823710. https://doi.org/10.4061/2010/823710 PMID:21188221

174. Hellings P, Jorissen M, Ceuppens JL. The Waldeyer's ring. Acta Otorhinolaryngol Belg. 2000; 54:237–41. PMID:11082757

175. Brandtzaeg P. Immunology of tonsils and adenoids: everything the ENT surgeon needs to know. Int J Pediatr Otorhinolaryngol. 2003 (Suppl 1); 67:S69–76. https://doi.org/10.1016/j.ijporl.2003.08.018 PMID:14662171

176. Fujimura Y, Akisada T, Harada T, Haruma K. Uptake of microparticles into the epithelium of human nasopharyngeal lymphoid tissue. Med Mol Morphol. 2006; 39:181–86. https://doi.org/10.1007/s00795-006-0335-6 PMID:17187179

177. Forchielli ML, Walker WA. The role of gut-associated lymphoid tissues and mucosal defence. Br J Nutr. 2005 (Suppl 1); 93:S41–48. https://doi.org/10.1079/bjn20041356 PMID:15877894

178. McGhee JR, Fujihashi K. Inside the mucosal immune system. PLoS Biol. 2012; 10:e1001397. https://doi.org/10.1371/journal.pbio.1001397 PMID:23049482

179. Faria AM, Garcia G, Rios MJ, Michalaros CL, Vaz NM. Decrease in susceptibility to oral tolerance induction and occurrence of oral immunization to ovalbumin in 20-38-week-old mice. The effect of interval between oral exposures and rate of antigen intake in the oral immunization. Immunology. 1993; 78:147–51. PMID:8436401

180. Hagiwara Y, McGhee JR, Fujihashi K, Kobayashi R, Yoshino N, Kataoka K, Etani Y, Kweon MN, Tamura S, Kurata T, Takeda Y, Kiyono H, Fujihashi K. Protective mucosal immunity in aging is associated with functional CD4+ T cells in nasopharyngeal-associated lymphoreticular tissue. J Immunol. 2003; 170:1754–62. https://doi.org/10.4049/jimmunol.170.4.1754 PMID:12574339

181. Koga T, McGhee JR, Kato H, Kato R, Kiyono H, Fujihashi K. Evidence for early aging in the mucosal immune system. J Immunol. 2000; 165:5352–59. https://doi.org/10.4049/jimmunol.165.9.5352 PMID:11046071

182. Klingenberg B, Hafkamp HC, Haesevoets A, Manni JJ, Slootweg PJ, Weissenborn SJ, Klussmann JP, Speel EJ. p16 INK4A overexpression is frequently detected in tumour-free tonsil tissue without association with HPV. Histopathology. 2010; 56:957–67. https://doi.org/10.1111/j.1365-2559.2010.03576.x PMID:20636796

183. Chen S, Wang WW, Wang Y, Li YQ, Zhu LX. Cellular senescence in recurrent tonsillitis and tonsillar hypertrophy in children. Int J Pediatr Otorhinolaryngol. 2020; 133:110004. https://doi.org/10.1016/j.ijporl.2020.110004 PMID:32200310

184. Yu Y, Park YS, Kim HS, Kim HY, Jin YM, Jung SC, Ryu KH, Jo I. Characterization of long-term *in vitro* culture-related alterations of human tonsil-derived mesenchymal stem cells: role for CCN1 in replicative senescence-associated increase in osteogenic differentiation. J Anat. 2014; 225:510–18. https://doi.org/10.1111/joa.12229 PMID:25155898

185. Choi DH, Oh SY, Choi JK, Lee KE, Lee JY, Park YJ, Jo I, Park YS. A transcriptomic analysis of serial-cultured, tonsil-derived mesenchymal stem cells reveals decreased integrin α3 protein as a potential biomarker of senescent cells. Stem Cell Res Ther. 2020; 11:359. https://doi.org/10.1186/s13287-020-01860-y PMID:32807231

186. Fujihashi K, Kiyono H. Mucosal immunosenescence: new developments and vaccines to control infectious diseases. Trends Immunol. 2009; 30:334–43. https://doi.org/10.1016/j.it.2009.04.004 PMID:19540811

187. Kawanishi H. Recent progress in senescence-associated gut mucosal immunity. Dig Dis. 1993; 11:157–72. https://doi.org/10.1159/000171408 PMID:8370142

188. Koyama K, Hosokawa T, Aoike A. Aging effect on the immune functions of murine gut-associated lymphoid tissues. Dev Comp Immunol. 1990; 14:465–73. https://doi.org/10.1016/0145-305x(90)90039-h PMID:2086275

189. Moretto MM, Lawlor EM, Khan IA. Aging mice exhibit a functional defect in mucosal dendritic cell response against an intracellular pathogen. J Immunol. 2008; 181:7977–84. https://doi.org/10.4049/jimmunol.181.11.7977 PMID:19017989

190. Kato H, Fujihashi K, Kato R, Dohi T, Fujihashi K, Hagiwara Y, Kataoka K, Kobayashi R, McGhee JR. Lack of oral tolerance in aging is due to sequential loss of Peyer's patch cell interactions. Int Immunol. 2003; 15:145–58. https://doi.org/10.1093/intimm/dxg011 PMID:12578844

191. Sreekumar PG, Hinton DR, Kannan R. The Emerging Role of Senescence in Ocular Disease. Oxid Med Cell Longev. 2020; 2020:2583601. https://doi.org/10.1155/2020/2583601 PMID:32215170

192. Bartling B. Cellular senescence in normal and premature lung aging. Z Gerontol Geriatr. 2013; 46:613–22. https://doi.org/10.1007/s00391-013-0543-3 PMID:23982440

193. Campisi J. The role of cellular senescence in skin aging. J Investig Dermatol Symp Proc. 1998; 3:1–5. PMID:9732048

194. Penfield JD, Anderson M, Lutzke L, Wang KK. The role of cellular senescence in the gastrointestinal mucosa. Gut Liver. 2013; 7:270–77. https://doi.org/10.5009/gnl.2013.7.3.270 PMID:23710306

195. Liu Y, Bloom SI, Donato AJ. The role of senescence, telomere dysfunction and shelterin in vascular aging. Microcirculation. 2019; 26:e12487. https://doi.org/10.1111/micc.12487 PMID:29924435

196. Minamino T, Komuro I. Vascular cell senescence: contribution to atherosclerosis. Circ Res. 2007; 100:15–26. https://doi.org/10.1161/01.RES.0000256837.40544.4a PMID:17204661

197. Fukuiwa T, Sekine S, Kobayashi R, Suzuki H, Kataoka K, Gilbert RS, Kurono Y, Boyaka PN, Krieg AM, McGhee JR, Fujihashi K. A combination of Flt3 ligand cDNA and CpG ODN as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. Vaccine. 2008; 26:4849–59. https://doi.org/10.1016/j.vaccine.2008.06.091 PMID:18625280

198. Dong Y, Lian X, Xu Y, Hu H, Chang C, Zhang H, Zhang L. Hematopoietic stem/progenitor cell senescence is associated with altered expression profiles of cellular memory-involved gene. Biosci Rep. 2018; 38:BSR20171589. https://doi.org/10.1042/BSR20171589 PMID:29363553

199. Kim HN, Chang J, Shao L, Han L, Iyer S, Manolagas SC, O'Brien CA, Jilka RL, Zhou D, Almeida M. DNA damage and senescence in osteoprogenitors expressing Osx1 may cause their decrease with age. Aging Cell. 2017; 16:693–703. https://doi.org/10.1111/acel.12597 PMID:28401730

200. Nakamura-Ishizu A, Suda T. Aging of the hematopoietic stem cells niche. Int J Hematol. 2014; 100:317–25. https://doi.org/10.1007/s12185-014-1641-8 PMID:25096220

201. Palmer DB. The effect of age on thymic function. Front Immunol. 2013; 4:316. https://doi.org/10.3389/fimmu.2013.00316 PMID:24109481

202. Turner VM, Mabbott NA. Structural and functional changes to lymph nodes in ageing mice. Immunology. 2017; 151:239–47. https://doi.org/10.1111/imm.12727 PMID:28207940

Research Paper

COVID-19 mortality rate in children is U-shaped

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ABSTRACT

Children are known to be better protected from COVID-19 than adults, but their susceptibility patterns and the risk relative to other diseases are insufficiently defined. Here, we found that the COVID-19 mortality rate is U-shaped in childhood: it initially decreases, reaching the minimum at the ages 3-10 years, and then increases throughout life. All-cause mortality and mortality from other diseases, such as pneumonia and influenza, show a similar pattern; however, childhood mortality rates from COVID-19 are considerably lower than from other diseases, with the best relative protection achieved at the youngest ages. Consistent with this, the fraction of COVID-19 deaths among all deaths increases as a function of age throughout childhood and the entire life. We discuss implications of the elevated postnatal COVID-19 risk and lower childhood COVID-19 mortality compared to other diseases.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a recently emerged coronavirus that causes the ongoing COVID-19 pandemic, originating from Wuhan, China, in December 2019 [1]. COVID-19 symptoms include coughing, shortness of breath, fever, fatigue, muscle aches, and more [2], but it targets multiple organs and is aggravated by diverse comorbidities. With nearly 200 million cases worldwide and more than four million deaths, COVID-19 remains a major public health threat.

COVID-19 primarily targets older individuals and those with comorbidities, whereas younger individuals are often spared [3]. Fewer children than adults develop severe pneumonia, and exhibit inflammatory markers, and many children infected by SARS-CoV-2 show no symptoms at all [3]. It was suggested that lower burden of COVID-19 in children [4] is due to decreased severity of infection, as is the case of several other infectious diseases, such as paralytic polio, rubella and severe respiratory distress syndrome (SARS) [5]. Interestingly, in newborns, COVID-19 may develop symptomatically, but vertical intrauterine transmission is rare [3].

Human all-cause mortality rate increases exponentially, with a doubling time of around 8 years [6]. However, the trend is opposite in early life where the mortality rate decreases from birth until the age of 9, where it reaches minimum, and then increases exponentially [7]. It is unclear how COVID-19 mortality changes in early life, whether it has a minimum, and whether children are better or worse protected from COVID-19 relative to other diseases. To fill this gap, we examined COVID-19 mortality data, revealing a U-shaped pattern of COVID-19 mortality and a considerably lower severity of this disease compared to other diseases in children.

RESULTS

U-shaped pattern of COVID-19 mortality rate

To understand how COVID-19 mortality changes over the entire lifespan, we analyzed the age-associated

mortality rate from COVID-19 in several countries (United States, United Kingdom and Spain), and compared it to the corresponding all-cause mortality. Age-associated COVID-19 mortality rate exhibited a U-shaped pattern, with the lowest rate observed at 3-10 years of age (Figure 1). A similar pattern was observed when the analysis was done for the entire world (Figure 2). As the absolute number of deceased subjects during early life is low and reporting patterns differed across countries, it was not possible to define the exact age at the minimum. However, all examined countries showed the U-shaped pattern, with the COVID-19 mortality rate in newborns and infants typically being as high as in 20-year-old subjects. All-cause mortality showed a similar U-shaped curve, with the minimum at the age of 9; however, the difference between COVID-19 mortality and all-cause mortality was considerably higher in young ages.

COVID-19 mortality shows the lowest rate in childhood compared to other related diseases

We analyzed childhood mortality rates for other diseases in the US and compared them to that of COVID-19 (Figure 3). Like COVID-19, influenza and pneumonia showed a U-shaped pattern that paralleled all-cause mortality. However, the COVID-19 mortality rate was disproportionally low in children under 12 (Figure 3A), suggesting a better protection against severe COVID-19 at the young ages compared to the two other infectious diseases. This could also be seen by the proportion of total deaths, which spiked at very early ages for pneumonia and influenza, but not for COVID-19 (Figure 3B). In adults over 20 years, the proportion of total deaths increased steadily with age for COVID-19 and pneumonia and then reached a plateau around 10% of all deaths. Interestingly, influenza showed a very different pattern, with a stable proportion well below 1% that did not increase with age.

Considering the common U-shaped mortality curves in early life for all analyzed diseases as well as for all-cause mortality, age emerged as an important risk factor for COVID-19 mortality from birth until the end of life. Additionally, we examined mortality rate from unspecified dementias, enteroviral infection, atherosclerotic heart disease, and sleep apnea (Figure 4). Their mortality patterns were also U-shaped, except for enteroviral infection, which had the highest mortality rate after birth and then decreased with age. These diseases were chosen to represent broader categories: enterovirus to cover the immune system and response [8], dementia to cover the nervous system, atherosclerosis and COVID-19 to cover the vascular system [9], and sleep apnea to cover the respiratory system. Even though enterovirus and the novel coronavirus represent infectious diseases, they exhibited remarkably different patterns, with enterovirus having the mortality rate minimum at the age of 40.

Children exhibit lower rates of severe symptoms than adults

We examined COVID-19 symptoms in children compared to adults (Table 1 and Figure 5), taking advantage of previously collected datasets [10, 11]. Adults showed higher rates of common symptoms than children, further supporting the protection that young ages offer against this disease. For example, 80% of adults had fever, compared to 59.9% of children, 84% of adults had cough versus 55.9% of children, and 38.4% of adults had rhinorrhea

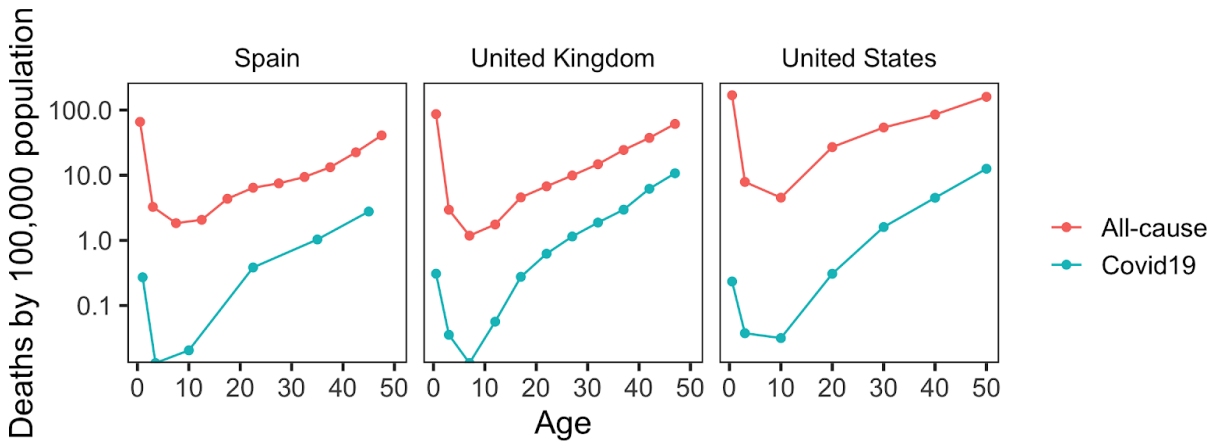

Figure 1. Age-related patterns of COVID-19 mortality. Data are shown for the indicated countries. Age-related change in all-cause mortality are also shown for these countries.

compared to 20% of children. Children also had unique symptoms in these studies, with nasal congestion being most frequent (20%), followed by sore throat (18.2%), and shortness of breath (11.7%) (Figure 5B). Some of these symptoms could be seen in adults in other studies, in particular, adults may experience shortness of breath during COVID-19 [12]. Additionally, there have been reports of adults having sore throat [13].

DISCUSSION

We found that COVID-19 shows a U-shaped pattern of age-associated mortality in several analyzed countries. The risk of dying from COVID-19 decreases during early life up to the age of 3-10 years, where it reaches its minimum, and then increases exponentially throughout life. The lowest rates were observed in 7-9 years old children in the US and UK, and 3-4 years old in Spain, but the exact minimum was difficult to pinpoint due to few cases of mortality at these ages. A similar pattern was found when we analyzed COVID-19 deaths across 37 countries based on COVerAGE-DB [14] (Figure 2). Although it is commonly believed that children are completely spared of COVID-19, our findings suggest that newborns and children during their first year of life exhibit a slightly elevated COVID-19 risk. Moreover, although it was not analyzed in this study, fetal mortality rate may be expected to be even higher. This is because COVID-19 follows a characteristic U-shaped mortality curve, also observed in the case of all-cause mortality and many diseases.

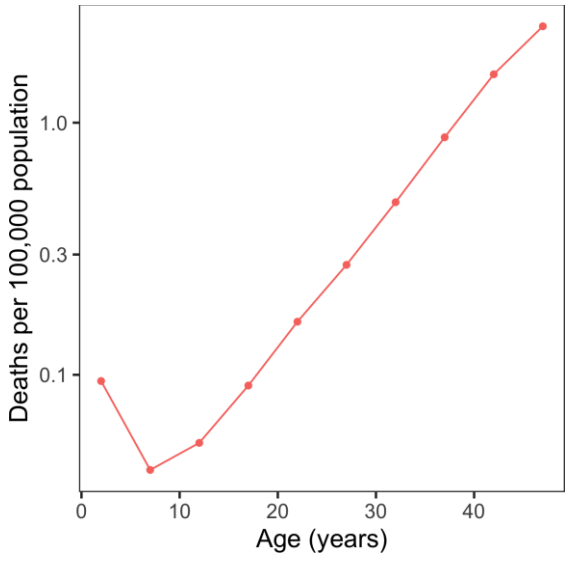

Figure 2. COVID-19 deaths per 100,000 population in the world. Shown is the world's COVID-19 mortality rate per 100,000 people per age interval.

Our second important finding is that children are disproportionately better protected from COVID-19 than from other common diseases analyzed in the study. While pneumonia and influenza also exhibited a U-shaped pattern, COVID-19 showed a lower mortality rate in children below the age of 12. COVID-19 mortality rate has previously been associated with aging [15], and analyses of infection rates [5] revealed a lower susceptibility in children. However, our study shows that COVID-19 susceptibility is not just lower in children than adults, but lower relative to many other diseases.

Some studies, such as a multinational cohort study in Europe [16], analyzed children with COVID-19 admitted to hospitals, and their observed patterns showed parallels with our results. The highest rate of ICU admission corresponded to the first month after birth, and the case-fatality rate for children was on average 0.69%. The most common source of infection was a parent or sibling.

Models that may help explain our findings are related to differences in the expression of ACE2, the SARS-CoV-2 receptor [17], and the lower burden of COVID-19 symptoms in children [3]. Children were found to express lower levels of ACE2 [17], while having a robust innate immune system to better deal with the virus during the entry point to the organism. Also, children may have had fewer opportunities to be infected due to the public health measures during the pandemic, such as school closures. It was also theorized that the impaired immune response could lead to higher COVID-19 vulnerability in adults [18], with older adults having a greater risk of mortality from infectious diseases that are targeted by common vaccines [19]. All these factors may lead to a much lower severity of COVID-19 in children except for newborns [10, 11]. Differences in COVID-19 symptoms may also related to gene expression patterns. The expression of genes IFNAR2 and TYK2 was found to be related to critical illness and severity of COVID-19 symptoms [20]. IFNAR2 is involved in innate antiviral defense, which is known to be important early in COVID-19. TYK2 is involved in host-driven inflammatory lung injury, which may lead to more severe symptoms [20, 21].

Another study analyzed disease severity and symptoms amongst children versus adults in China [21] and found that the majority (66.7%) of children in Jinan Infectious Diseases Hospital exhibited no symptoms. None of the children in the study needed a ventilator or additional accommodations. However, it reported a high level of creatinine kinase-MB (CK-MB; an indicator of myocardial injury) in most

children versus few adults, suggesting that children may exhibit higher rates of myocardial injury caused by the virus. Our analyses also revealed symptoms that were more common in children. The symptoms common to both children and adults were generally less severe in children. An additional study that examined symptoms in children had similar findings [22], e.g. it was found that respiratory symptoms were generally mild, with fever being the most common symptom amongst children.

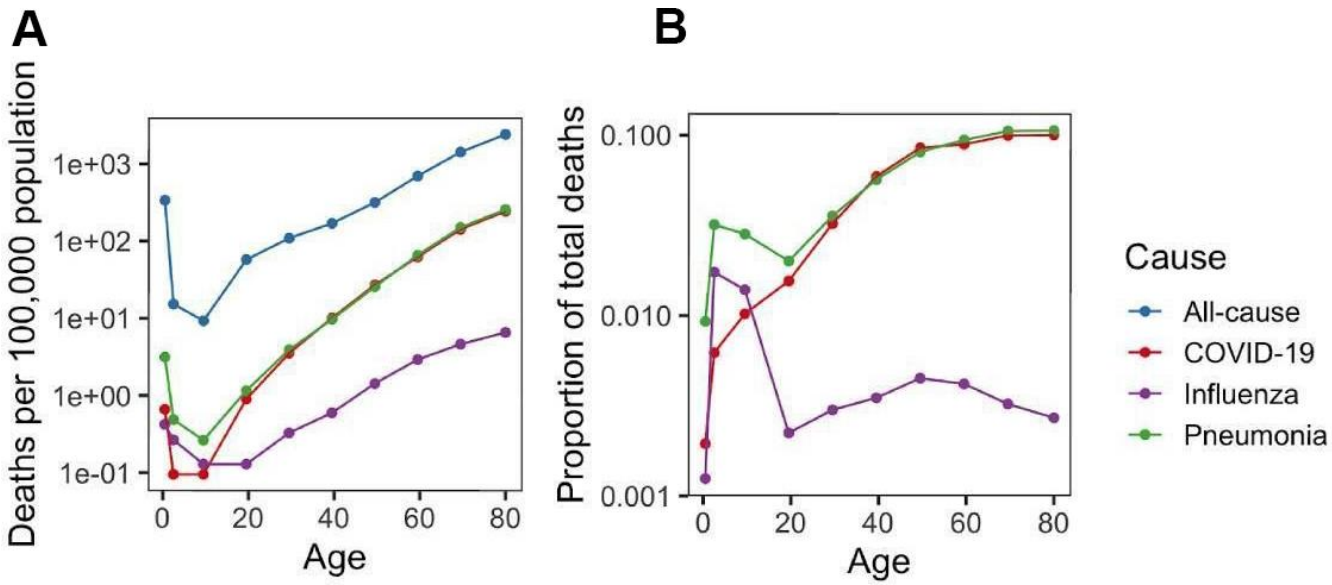

Figure 3. Burden of COVID-19, pneumonia and influenza. (**A**) Mortality rate (per 100,000 population) for COVID-19, pneumonia and influenza as well as all-cause mortality rate. (**B**) Ratio of COVID-19, pneumonia and influenza mortality rate to all-cause mortality rate.

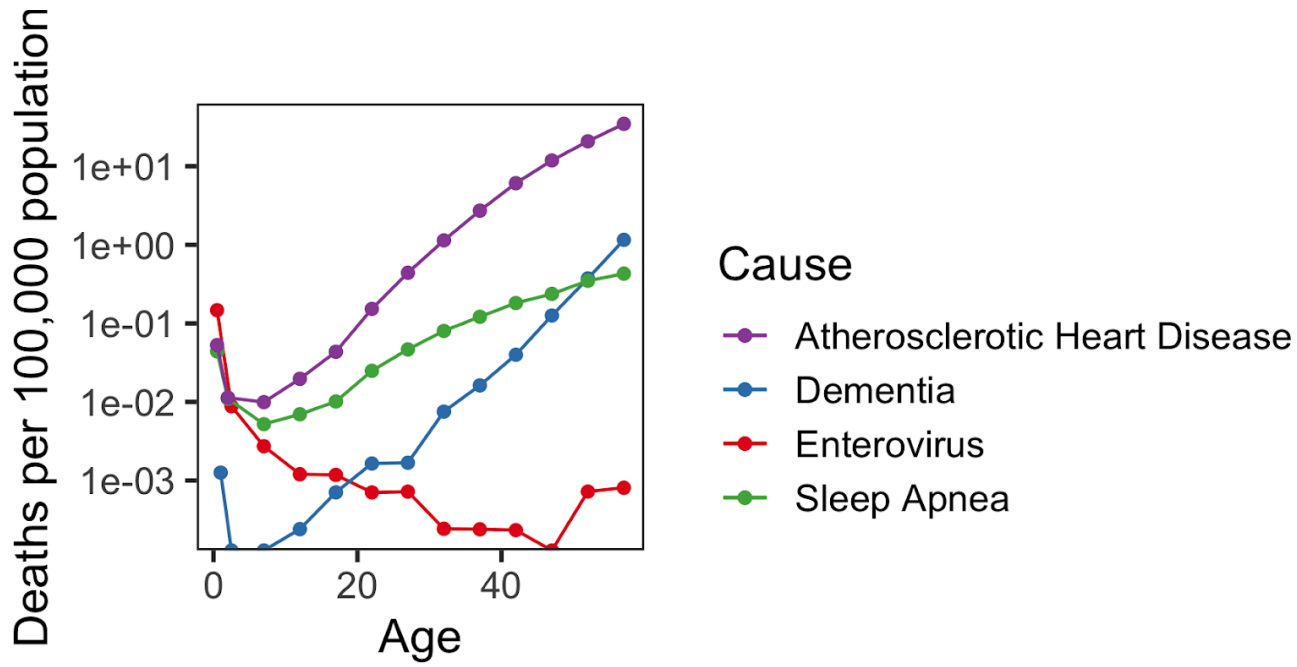

Figure 4. Mortality rate from other diseases. Mortality rate (per 100,000 population) across lifespan is shown for atherosclerotic heart disease, dementia, sleep apnea, and enteroviral infection.

Table 1. Frequency of COVID-19 symptoms in US children and adults.

Symptoms	Children in the US (%)	Adults in the US (%)
Fever of at least 37.5 degrees	59.1	80
Cough	55.9	84
Rhinorrhea	20	38.4
Nasal congestion	20	N/A
Fatigue	18.7	62
Myalgia	18.7	63
Sore Throat	18.2	N/A
Shortness of breath	11.7	N/A
Diarrhea	6.5	38
Abdominal pain	6.5	N/A
Nausea	5.4	N/A
Vomiting	5.4	13
Headaches	4.3	59
Dizziness	4.3	N/A
Pharyngeal Erythema	3.3	N/A
Decreased oral intake	1.7	N/A
Rash	0.25	N/A
Chills	N/A	63
Tachypnoea	N/A	57
Changes in smell or taste	N/A	13.9

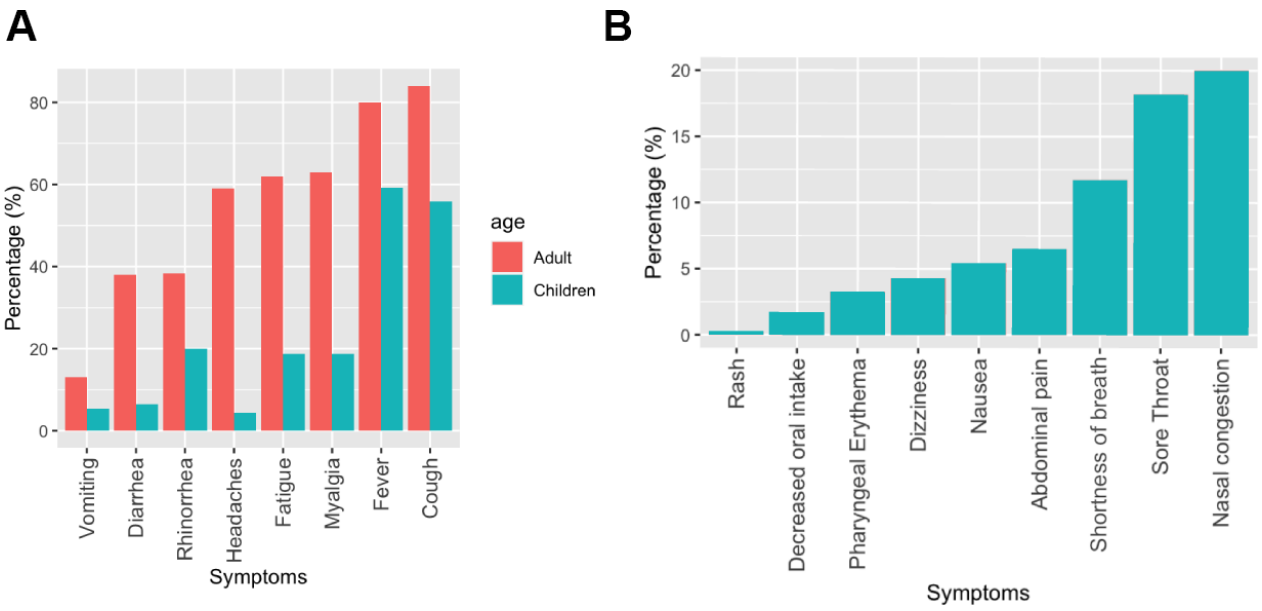

Figure 5. Frequency of COVID-19 symptoms in children compared to adults. (**A**) Frequency of common COVID-19 symptoms in children and adults. (**B**) Rank order of COVID-19 symptoms specific to children.

Limitations of our study include the fact that it was restricted to a relatively small number of COVID-19 cases in children due to the novelty of the disease. As we did not consider ethnicity or gender, additional studies are needed to address their relevance [23]. Along these lines, we examined the fraction of total deaths related to COVID-19 in the US separately for the two sexes (Figure 6). The most prominent difference was around the age 20, which is consistent with riskier behavior by men at these ages. An interesting area to explore is the impact of a previous COVID-19 infection on children's overall health and long-term wellness. A case report of 5 COVID-19 cases in children in Sweden showed that 6-8 months after infection, they stopped experiencing most of the symptoms, but their fatigue persisted [24].

Overall, our study establishes that the COVID-19 mortality rate is U-shaped, like that of pneumonia, influenza and many other diseases. Thus, newborns have a somewhat increased risk of severe COVID-19, and children below the ages 1-3 years exhibit elevated COVID-19 burden compared to older children. On the other hand, children in general are greatly spared from COVID-19 compared to other common diseases. These pronounced age-related patterns suggest that the factors that affect biological age may influence COVID-19 infection and severity. Thus, while COVID-19 is known to be most dangerous for the elderly and those with chronic diseases, children are also affected, with the perinatal period being more dangerous that later childhood. Finally, our study suggests the observed age-related patterns of COVID-19 susceptibility in children should be considered when developing COVID-19 regulations.

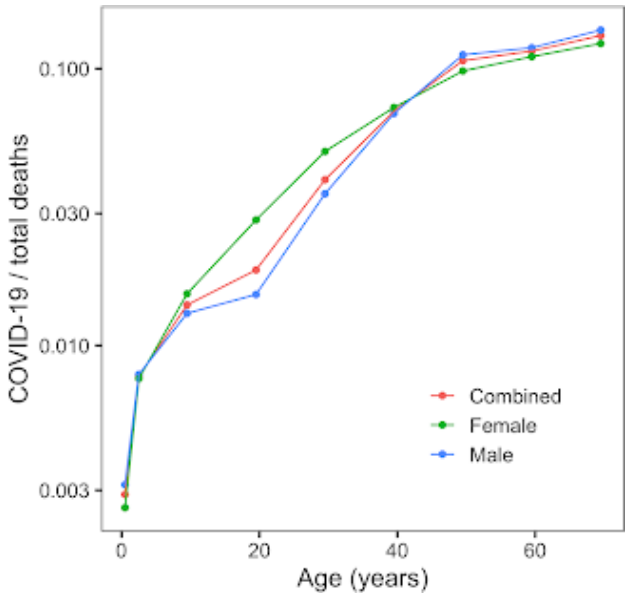

Figure 6. Fraction of total deaths in the United States attributable to COVID-19. Fraction of total deaths, sorted by age and color-coded by sex, of total recorded deaths in the United States that had their cause pinpointed as COVID-19.

MATERIALS AND METHODS

Data sources

United States data on confirmed COVID-19 cases and hospitalizations categorized by age were from the Center for Disease Control [25]. We used the Office for National Statistics' data on all-cause mortality, reported weekly [26], as well as their data on the population in different regions of the UK by age [27]. These were used for the analysis of fatality rates in the US and the UK. As a reference, we used statistics of 'Our world in data' on all-cause infant and child mortality [28]. We computed deaths per 100,000 population. For the same analysis with Spain, we used Insitituto de Salud Carlos III's data on COVID-19 cases and fatalities [29]. Data from the United Nations on population was used to inform many of the population-related calculations [30]. Additionally, data from hospitals was used to comparatively analyze symptomal frequencies [10, 11]. The data was last searched December of 2020. There is no review protocol.

Calculation of COVID-19 mortality rates

Total COVID-19 deaths by 100,000 population were calculated for Spain, the UK, and USA using data on COVID-19 deaths overall and population from various governmental centers and hospitals [25–27, 29], and plotted using ggplot in R. COVID-19 case-fatality ratios per 100,000 people were calculated based on COVerAGE [14] and plotted using ggplot in R.

Comparison of mortality from COVID-19, pneumonia and influenza

Mortality rate was calculated for different diseases, as well as all-cause mortality rate, and the ratios of the mortality rates from the aforementioned diseases to all-cause mortality rate were computed. This was done using data on deaths from these different diseases, all-cause deaths, and population within the US from the CDC and the government overall [25, 30].

Mortality from other diseases

Mortality rate was calculated for various diseases, as well as all-cause mortality rate, and the ratios of the mortality rates from the aforementioned diseases to all-cause mortality rate were computed. This was done using data on deaths from these diseases, all-cause

deaths, and population within the US from the CDC Wonder Database [31].

Analysis of COVID-19 symptoms in children compared to adults

Multiple studies of percentages of symptoms in children and adults were examined, and statistics that were found per symptom were plotted, comparing both children and adults, and children overall. The data was retrieved from official hospital studies [10, 11]. Everything was plotted using ggplot in R. For Table 1, the aforementioned symptomal statistics were added. In some datasets, data was presented as part of different weeks. When this was the case, all relevant data was added and compiled.

AUTHOR CONTRIBUTIONS

N.K. carried out the analyses. D.S. and C.K contributed to data analyses and supervision. V.N.G. conceived and supervised the project. N.K. and D.S. created figures. The manuscript was written by N.K., C.K. and V.N.G.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Ciotti M, Angeletti S, Minieri M, Giovannetti M, Benvenuto D, Pascarella S, Sagnelli C, Bianchi M, Bernardini S, Ciccozzi M. COVID-19 Outbreak: An Overview. Chemotherapy. 2019; 64:215–23. https://doi.org/10.1159/000507423 PMID:32259829

2. Rothan HA, Byrareddy SN. The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. J Autoimmun. 2020; 109:102433. https://doi.org/10.1016/j.jaut.2020.102433 PMID:32113704

3. Ludvigsson JF. Systematic review of COVID-19 in children shows milder cases and a better prognosis than adults. Acta Paediatr. 2020; 109:1088–95. https://doi.org/10.1111/apa.15270 PMID:32202343

4. Garg S, Kim L, Whitaker M, O'Halloran A, Cummings C, Holstein R, Prill M, Chai SJ, Kirley PD, Alden NB, Kawasaki B, Yousey-Hindes K, Niccolai L, et al. Hospitalization Rates and Characteristics of Patients Hospitalized with Laboratory-Confirmed Coronavirus Disease 2019 - COVID-NET, 14 States, March 1-30, 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:458–64. https://doi.org/10.15585/mmwr.mm6915e3 PMID:32298251

5. Lee PI, Hu YL, Chen PY, Huang YC, Hsueh PR. Are children less susceptible to COVID-19? J Microbiol Immunol Infect. 2020; 53:371–72. https://doi.org/10.1016/j.jmii.2020.02.011 PMID:32147409

6. Finch CE. Longevity, Senescence, and the Genome. Chicago, London: University of Chicago Press. 1990.

7. Kinzina ED, Podolskiy DI, Dmitriev SE, Gladyshev VN. Patterns of Aging Biomarkers, Mortality, and Damaging Mutations Illuminate the Beginning of Aging and Causes of Early-Life Mortality. Cell Rep. 2019; 29:4276–84.e3. https://doi.org/10.1016/j.celrep.2019.11.091 PMID:31875539

8. Bopegamage S. Enterovirus infections: Pivoting role of the adaptive immune response. Virulence. 2016; 7:495–97. https://doi.org/10.1080/21505594.2016.1175701 PMID:27058267

9. Siddiqi HK, Libby P, Ridker PM. COVID-19 - A vascular disease. Trends Cardiovasc Med. 2021; 31:1–5. https://doi.org/10.1016/j.tcm.2020.10.005 PMID:33068723

10. Hoang A, Chorath K, Moreira A, Evans M, Burmeister-Morton F, Burmeister F, Naqvi R, Petershack M, Moreira A. COVID-19 in 7780 pediatric patients: A systematic review. EClinicalMedicine. 2020; 24:100433. https://doi.org/10.1016/j.eclinm.2020.100433 PMID:32766542

11. Burke RM, Killerby ME, Newton S, Ashworth CE, Berns AL, Brennan S, Bressler JM, Bye E, Crawford R, Harduar Morano L, Lewis NM, Markus TM, Read JS, et al, and Case Investigation Form Working Group. Symptom Profiles of a Convenience Sample of Patients with COVID-19 - United States, January-April 2020. MMWR Morb Mortal Wkly Rep. 2020; 69:904–08. https://doi.org/10.15585/mmwr.mm6928a2 PMID:32673296

12. Cortés-Telles A, López-Romero S, Figueroa-Hurtado E, Pou-Aguilar YN, Wong AW, Milne KM, Ryerson CJ, Guenette JA. Pulmonary function and functional capacity in COVID-19 survivors with persistent dyspnoea. Respir Physiol Neurobiol. 2021; 288:103644. https://doi.org/10.1016/j.resp.2021.103644 PMID:33647535

13. Grant MC, Geoghegan L, Arbyn M, Mohammed Z, McGuinness L, Clarke EL, Wade RG. The prevalence of

symptoms in 24,410 adults infected by the novel coronavirus (SARS-CoV-2; COVID-19): A systematic review and meta-analysis of 148 studies from 9 countries. PLoS One. 2020; 15:e0234765. https://doi.org/10.1371/journal.pone.0234765 PMID:32574165

14. Riffe T, Acosta E, and the COVerAGE-DB team. Data Resource Profile: COVerAGE-DB: a global demographic database of COVID-19 cases and deaths. Int J Epidemiol. 2021; 50: 390-90f. https://doi.org/ 10.1093/ije/dyab027

15. Santesmasses D, Castro JP, Zenin AA, Shindyapina AV, Gerashchenko MV, Zhang B, Kerepesi C, Yim SH, Fedichev PO, Gladyshev VN. COVID-19 is an emergent disease of aging. Aging Cell. 2020; 19:e13230. https://doi.org/10.1111/acel.13230 PMID:33006233

16. Götzinger F, Santiago-García B, Noguera-Julián A, Lanaspa M, Lancella L, Calò Carducci FI, Gabrovska N, Velizarova S, Prunk P, Osterman V, Krivec U, Lo Vecchio A, Shingadia D, et al, and ptbnet COVID-19 Study Group. COVID-19 in children and adolescents in Europe: a multinational, multicentre cohort study. Lancet Child Adolesc Health. 2020; 4:653–61. https://doi.org/10.1016/S2352-4642(20)30177-2 PMID:32593339

17. Vaduganathan M, Vardeny O, Michel T, McMurray JJ, Pfeffer MA, Solomon SD. Renin-Angiotensin-Aldosterone System Inhibitors in Patients with Covid-19. N Engl J Med. 2020; 382:1653–59. https://doi.org/10.1056/NEJMsr2005760 PMID:32227760

18. Su L, Ma X, Yu H, Zhang Z, Bian P, Han Y, Sun J, Liu Y, Yang C, Geng J, Zhang Z, Gai Z. The different clinical characteristics of corona virus disease cases between children and their families in China - the character of children with COVID-19. Emerg Microbes Infect. 2020; 9:707–13. https://doi.org/10.1080/22221751.2020.1744483 PMID:32208917

19. Suratannon N, Dik WA, Chatchatee P, Hagen PM. COVID-19 in children: Heterogeneity within the disease and hypothetical pathogenesis. Asian Pac J Allergy Immunol. 2020; 38:170–77. https://doi.org/10.12932/AP-170720-0920 PMID:32990448

20. Pairo-Castineira E, Clohisey S, Klaric L, Bretherick AD, Rawlik K, Pasko D, Walker S, Parkinson N, Fourman MH, Russell CD, Furniss J, Richmond A, Gountouna E, et al, and GenOMICC Investigators, and ISARIC4C Investigators, and COVID-19 Human Genetics Initiative, and 23andMe Investigators, and BRACOVID Investigators, and Gen-COVID Investigators. Genetic mechanisms of critical illness in COVID-19. Nature. 2021; 591:92–98. https://doi.org/10.1038/s41586-020-03065-y PMID:33307546

21. Berg J, Zscheppang K, Fatykhova D, Tönnies M, Bauer TT, Schneider P, Neudecker J, Rückert JC, Eggeling S, Schimek M, Gruber AD, Suttorp N, Hippenstiel S, Hocke AC. Tyk2 as a target for immune regulation in human viral/bacterial pneumonia. Eur Respir J. 2017; 50:1601953. https://doi.org/10.1183/13993003.01953-2016 PMID:28705941

22. Castagnoli R, Votto M, Licari A, Brambilla I, Bruno R, Perlini S, Rovida F, Baldanti F, Marseglia GL. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection in Children and Adolescents: A Systematic Review. JAMA Pediatr. 2020; 174: 882–89. https://doi.org/10.1001/jamapediatrics.2020.1467 PMID:32320004

23. Jin JM, Bai P, He W, Wu F, Liu XF, Han DM, Liu S, Yang JK. Gender Differences in Patients With COVID-19: Focus on Severity and Mortality. Front Public Health. 2020; 8:152. https://doi.org/10.3389/fpubh.2020.00152 PMID:32411652

24. Ludvigsson JF. Case report and systematic review suggest that children may experience similar long-term effects to adults after clinical COVID-19. Acta Paediatr. 2021; 110:914–21. https://doi.org/10.1111/apa.15673 PMID:33205450

25. COVIDView: A Weekly Surveillance Summary of U.S. COVID-19 Activity. Centers for Disease Control and Prevention. 2021. https://www.cdc.gov/coronavirus/2019-ncov/covid-data/covidview/index.html

26. Deaths registered weekly in England and Wales, provisional. Deaths registered weekly in England and Wales, provisional. Office for National Statistics. 2021. https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/datasets/weeklyprovisionalfiguresondeathsregisteredinenglandandwales

27. Estimates of the population for the UK, England and Wales, Scotland and Northern Ireland. Office for National Statistics. 2020. https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/datasets/populationestimatesforukenglandandwalesscotlandandnorthernireland

28. Roser M, Ritchie H, Dadonaite B. Child and Infant Mortality. Our World in Data. 2013. https://ourworldindata.org/child-mortality

29. ISCIII. Informes COVID-19. 2020. https://www.

isciii.es/QueHacemos/Servicios/VigilanciaSaludPublicaRENAVE/EnfermedadesTransmisibles/Paginas/InformesCOVID-19.aspx

30. World Population Prospects - Population Division. United Nations. 2020. https://population.un.org/wpp/Download/Standard/CSV/

31. Centers for Disease Control and Prevention, National Center for Health Statistics. Multiple Cause of Death 1999-2018 on CDC WONDER Online Database, released in 2020. Data are from the Multiple Cause of Death Files, 1999-2018, as compiled from data provided by the 57 vital statistics jurisdictions through the Vital Statistics Cooperative Program. 2020. http://wonder.cdc.gov/mcd-icd10.html

Priority Research Paper

SARS-CoV-2 causes senescence in human cells and exacerbates the senescence-associated secretory phenotype through TLR-3

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ABSTRACT

Senescent cells, which arise due to damage-associated signals, are apoptosis-resistant and can express a pro-inflammatory, tissue-destructive senescence-associated secretory phenotype (SASP). We recently reported that a component of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) surface protein, S1, can amplify the SASP of senescent cultured human cells and that a related mouse β-coronavirus, mouse hepatitis virus (MHV), increases SASP factors and senescent cell burden in infected mice. Here, we show that SARS-CoV-2 induces senescence in human non-senescent cells and exacerbates the SASP in human senescent cells through Toll-like receptor-3 (TLR-3). TLR-3, which senses viral RNA, was increased in human senescent compared to non-senescent cells. Notably, genetically or pharmacologically inhibiting TLR-3 prevented senescence induction and SASP amplification by SARS-CoV-2 or Spike pseudotyped virus. While an artificial TLR-3 agonist alone was not sufficient to induce senescence, it amplified the SASP in senescent human cells. Consistent with these findings, lung p16^{INK4a+} senescent cell burden was higher in patients who died from acute SARS-CoV-2 infection than other causes. Our results suggest that induction of cellular senescence and SASP amplification through TLR-3 contribute to SARS-CoV-2 morbidity, indicating that clinical trials of senolytics and/or SASP/TLR-3 inhibitors for alleviating acute and long-term SARS-CoV-2 sequelae are warranted.

INTRODUCTION

The current COVID-19 pandemic has illuminated the vulnerability of the elderly and those with chronic diseases to increased SARS-CoV-2-mediated mortality. By August 2021, there had been over 207 million cases of SARS-CoV-2 and 4.3 million deaths worldwide (https://en.wikipedia.org/wiki/Template:COVID-19_pandemic_data accessed 08/16/2021). Those over age 65 accounted for 45% of patients hospitalized and 80% of those who died with SARS-CoV-2 [1]. Recent studies have estimated that between 10-30% of patients experience persistent symptoms months after resolution of acute cases of COVID-19. These prolonged symptoms are referred to as post-acute sequelae of SARS-CoV-2 infection (PASC) and might be a consequence of chronic inflammation induced during the acute phase of infection [2]. A mechanistic level of understanding of the short- and long-term effects of SARS-CoV-2 infection on cell and tissue function is urgently needed to tackle its acute and chronic adverse health outcomes.

We recently reported that senescent cells contribute to the pathogenesis of acute β-coronavirus infections [3]. The senescent cell fate entails essentially permanent cell-cycle arrest with extensive changes in cell morphology and gene expression [4–6]. Senescence in many cell types is driven by damage/danger signals as well as metabolic insults, mechanical/shear forces, hypoxia, reactive oxygen species (ROS), repeated replication, oncogenes, telomere damage, and other stressors [7–10]. Senescence is established through transcription factor cascades that can include $p16^{INK4a}$/retinoblastoma protein and/or $p53/p21^{CIP1}$, which induce extensive changes in gene expression and organelle function, histone modifications, epigenomic remodelling, altered protein production, and profound morphologic and metabolic shifts [11, 12]. Senescent cells are resistant to apoptosis, persistent, metabolically active, and mainly cleared by the immune system [13–15]. A majority of, but not all senescent cells can develop a senescence-associated secretory phenotype (SASP), with release of inflammatory cytokines, chemokines, proteases, pro-coagulant and pro-fibrotic factors, bioactive lipids, other reactive metabolites, non-coding nucleotides, and extracellular vesicles [16–19]. The SASP can induce secondary senescence of neighboring previously non-senescent cells and even of cells at a distance [20, 21].

Senescence and the SASP have emerged as mechanisms that appear to contribute to aging phenotypes and multiple chronic conditions and diseases, even in younger individuals (*e.g.*, in those with obesity/diabetes, cardiac, lung, and kidney disorders, arthritis, cancers, osteoporosis, or neurocognitive or immunological dysfunction), and can underlie adverse effects due to certain drugs and treatments, such as chemotherapy or radiation [5, 22–32]. Many of the clinical conditions linked to cellular senescence share features with complications associated with sequelae of COVID-19. Both can be associated with cognitive dysfunction, frailty/weakness, arthritis and arthralgias, cardiac conditions, and lung dysfunction and fibrosis, among others [4, 5, 23, 24, 27, 29, 31, 33–35]. Additionally, factors such as IL-6, IL-8, and IP-10 that are SASP components appear to predict the severity of SARS-CoV-2 infection [36]. These factors may also contribute to prolonged disease, hyper-inflammation/cytokine storm, acute respiratory distress (ARDS), and multi organ failure.

Incoming viral pathogens are detected by the innate immune system through dedicated sensors, Toll-Like Receptors (TLRs), which recognize pathogen-associated molecular patterns and engage innate immune responses. Although SASP-related cytokines are a critical component of the innate immune response and aid in clearing viral infections, dysregulated release of pro-inflammatory cytokines may lead to cytokine storm, which can result in severe damage to host tissues and organs. Here, we examined innate immune responses to SAR-CoV-2 and links to cellular senescence. We found that both a Spike pseudotyped virus (pseudovirus) and the genuine SARS-CoV-2 virus can induce senescence in human cells. Furthermore, the senescent cell SASP was amplified by TLR-3-dependent signaling. Clinical trials appear be warranted to ascertain if senolytics, agents that selectively eliminate senescent cells, senomorphics, which inhibit the SASP, and/or TLR-3 inhibitors can alleviate acute or long-term sequelae of SARS-CoV-2 infection.

RESULTS

Increased TLR-3 expression in senescent cells

TLRs are major innate immune sensors whose expression varies depending on cell type [37]. Therefore, we analyzed if TLRs are present on senescent cells. TLR-1, -3, and -4 were more highly expressed in radiation-induced senescent *vs.* non-senescent human preadipocytes (Figure 1A). Among other ligands, TLR-1 binds gram-positive bacterial antigens, TLR-3 binds viral RNA, and TLR-4 binds lipopolysaccharide [38]. Also, TLR-3 mRNA was increased in senescent human kidney endothelial cells compared to non-senescent controls (Figure 1B). TLR-3 protein levels were higher in radiation-induced senescent human kidney endothelial cells than non-senescent controls (Figure 1C). Thus, TLR-3, a sensor of viral RNA, can be present in increased abundance on senescent cells.

Pseudovirus induces senescence in non-senescent human kidney endothelial and lung epithelial cells

We previously reported that mouse coronavirus induces senescence *in vivo* (see Figure 3B) [3]. To examine whether exposure of senescent cells to SARS-CoV-2 might induce senescence through TLR-3, non-senescent human cells were exposed to vesicular stomatitis virus (VSV) encoding SARS-CoV-2 Spike instead of its native G Glycoprotein (VSVdG*Spike), a pseudovirus that simulates the presence of SARS-CoV-2 but is safer to handle [39]. Expression of the cellular senescence markers $p16^{INK4a}$ and $p21^{CIP1}$ was increased by exposing non-senescent human kidney endothelial (Figure 2A) and lung epithelial cells (Figure 2B) to this pseudovirus for 14 days. The TLR-3 antagonist, (R)-2-(3-chloro-6-fluorobenzo[b]thiophene-2-carboxamido)-3-phenylpropanoic acid, counteracted this induction of senescence markers by the pseudovirus in both human kidney endothelial and lung epithelial cells (Figure 2A, 2B). Exposure to a TLR-3 agonist, polyinosine-polycytidylic acid (Poly I:C), for 1 week tended to induce increase in $p16^{INK4a}$ and $p21^{CIP1}$ expression in human non-senescent preadipocytes, but not significantly (Figure 2C). Taken together, these results indicate that pseudovirus sensed by TLR-3 is sufficient to promote senescence.

Pseudovirus amplifies the SASP of senescent human cells *via* TLR-3

Sensing of a virus by TLR-3 usually induces pro-inflammatory cytokines, including IL-6, MCP-1, and TNFα. To assess whether SARS-CoV-2 sensing by TLR-3 promotes the release of pro-inflammatory cytokines and amplifies the SASP, senescent human preadipocytes (Figure 3A) and senescent kidney endothelial cells (Supplementary Figure 2) were exposed to the pseudovirus for 96 hrs as well to non-senescent control cells. After exposure, pseudovirus-exposed senescent cells had increased expression of the key SASP

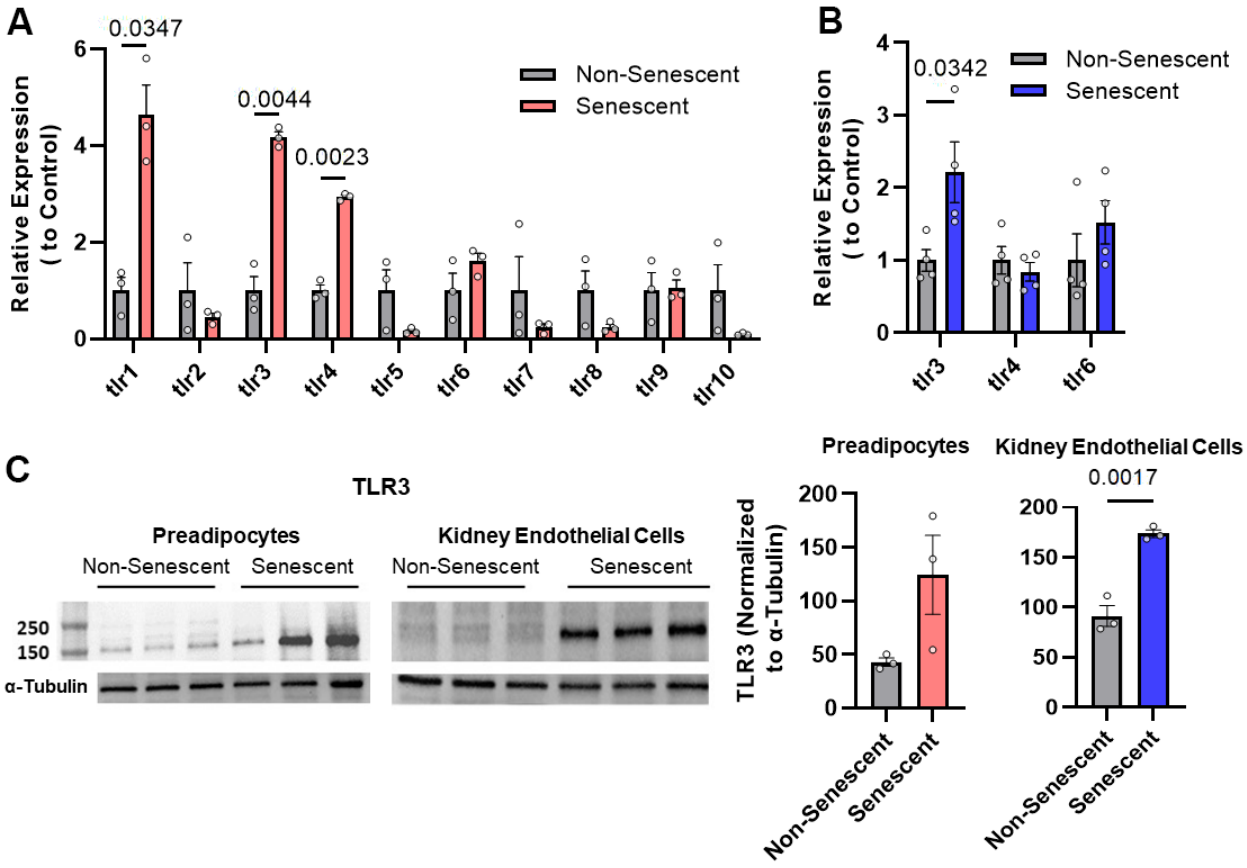

Figure 1. Toll-like receptor-3 (TLR-3) is increased in senescent *vs*. non-senescent human kidney endothelial cells and preadipocytes. (**A**) TLR expression (rtPCR) in radiation-induced senescent *vs*. non-senescent human preadipocytes (n=3). (**B**) TLR (rtPCR) in radiation-induced senescent human kidney endothelial cells (n=4) *vs*. non-senescent cells. Data are expressed as a function of non-senescent cells; mean +/- SEM, paired (**A**), unpaired (**B**), 2-tailed Student's t-tests. (**C**) TLR-3 protein (Western blots) in non-senescent and senescent preadipocytes (n=3) and kidney endothelial cells (n=3) with tubulin as loading control (optical density of TLR-3 as a function of α-tubulin [%]). Data are expressed as a function of non-senescent cells; mean +/- SEM, paired (preadipocytes), unpaired (kidney endothelial cells), 2-tailed Student's t-tests.

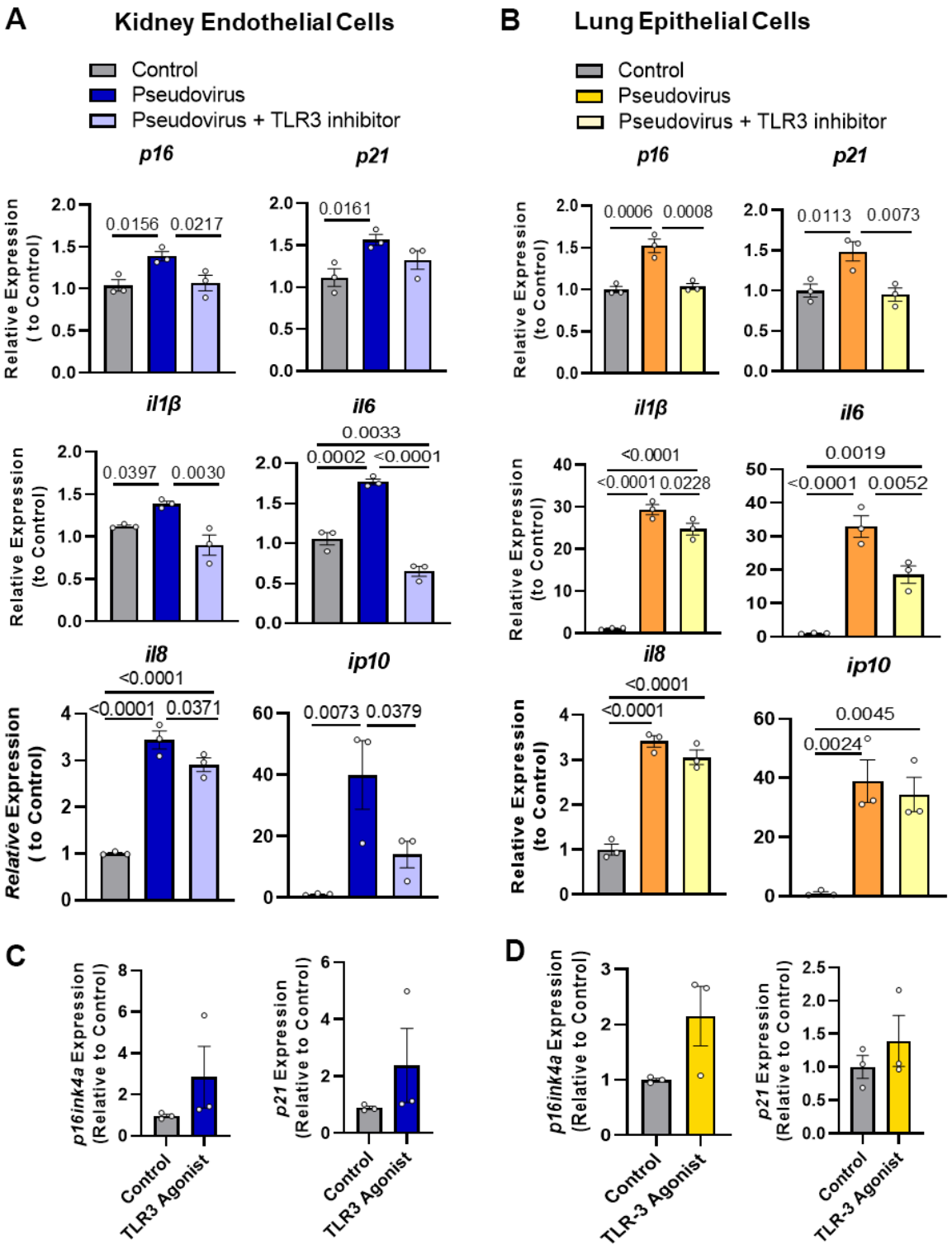

Figure 2. Pseudovirus exposure increases senescence markers in non-senescent cells; these increases in markers were attenuated by TLR-3 inhibitor. (**A**) Non-senescent kidney endothelial cells and (**B**) human lung epithelial cells had higher expression of senescence markers and SASP factors upon treatment with pseudovirus, while using TLR-3 inhibitor decreased their expression. Cells were exposed to the pseudovirus in the presence of TLR-3 inhibitor or vehicle (control) for 14 days. (**C**, **D**) Activating TLR-3 was not sufficient to induce senescence: TLR-3 agonist did not induce senescence as extensively as the pseudovirus. Non-senescent kidney endothelial (**C**) and lung epithelial cells (**D**) were treated with the TLR-3 agonist Poly I:C (2 and 10μg/ml, respectively), for 7 days or pseudovirus, when senescence markers and SASP factor expression were measured. Data are expressed as a function of untreated non-senescent cells; mean +/- SEM, 1-way ANOVA and *post hoc* comparisons with Fisher's LSD (**A**, **B**) and unpaired Student's t-tests (**C**, **D**).

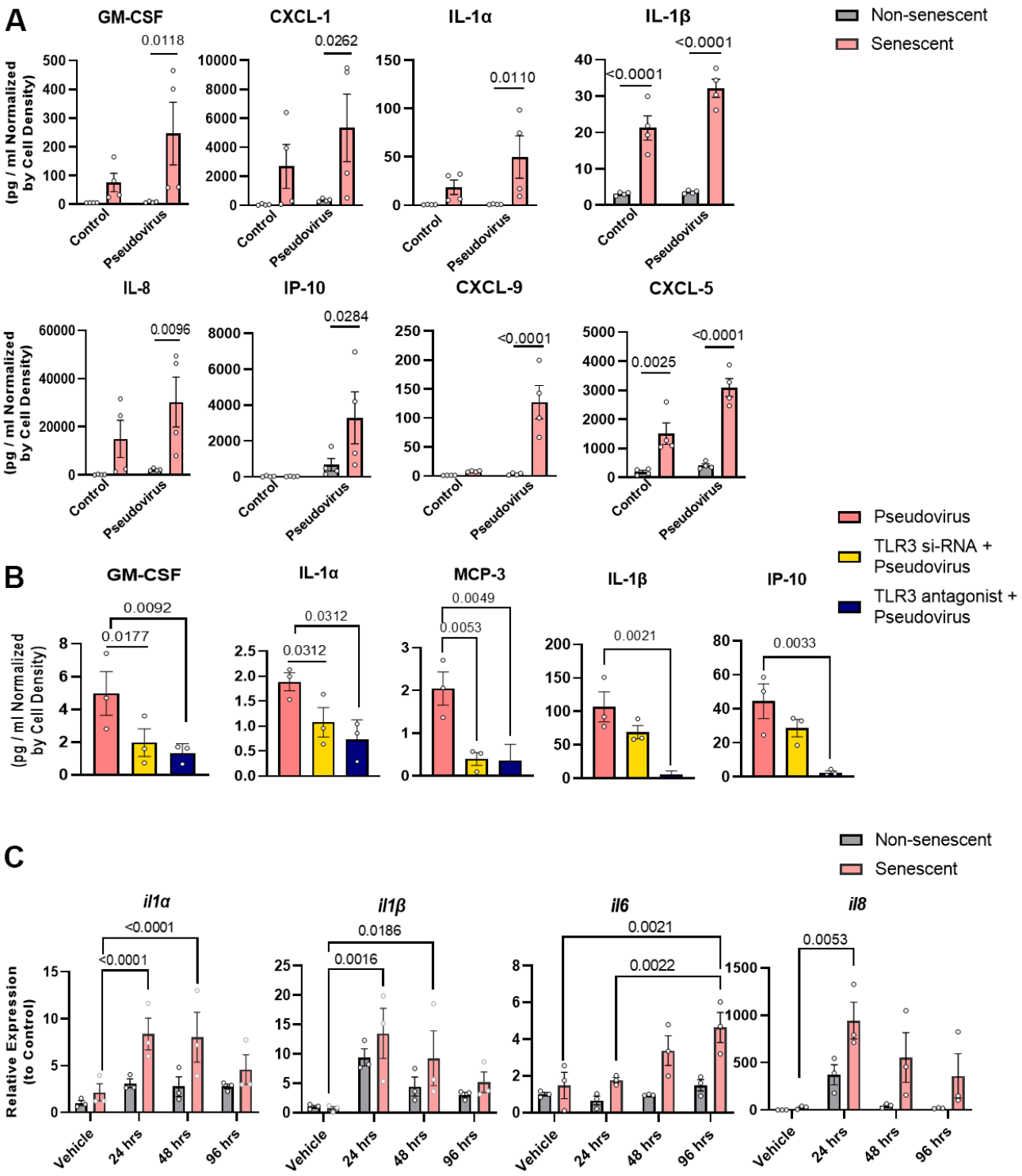

Figure 3. Pseudovirus amplifies the SASP in senescent preadipocytes and genetically or pharmacologically inhibiting TLR-3 attenuates this SASP amplification. (**A**) SASP factors were assayed in senescent and non-senescent preadipocytes (n=4) treated with pseudovirus for 96 hrs. Data are shown as a function of cell number; mean +/- SEM, 2-way repeated measures ANOVA. (**B**) Senescent cells were transfected with TLR-3 siRNA or treated with TLR-3 antagonist (10μM) and then exposed to pseudovirus for 96 hrs. Data are expressed as a function of untreated senescent cells; mean +/- SEM, repeated 1-way ANOVA and *post hoc* pairwise comparisons Fischer's LSD. (**C**) TLR-3 agonist increases SASP factor expression in senescent (but not non-senescent) human preadipocytes. Senescent and non-senescent preadipocytes were exposed to Poly I:C for 24, 48, or 96 hrs. and analyzed for SASP factors (rtPCR). Data are expressed as a function of untreated senescent cells; mean +/- SEM, repeated 2-way ANOVA and *post hoc* pairwise comparison Tukey's HSD. All other significant p values are listed in Supplementary Table 1.

factors, IL-1α, IL-1β, IL-8, and chemokine-like CXCL5, compared to non-senescent preadipocytes. This amplification was attenuated by decreasing TLR-3 expression using siRNAs or by treatment with a TLR-3 antagonist (Figure 3B and Supplementary Figure 1). This suggests that TLR-3 activation is sufficient for the pseudovirus to amplify the pro-inflammatory SASP of pre-existing senescent cells.

To determine if senescent cells respond directly to TLR-3 activation, radiation-induced senescent human preadipocytes *vs.* non-exposed senescent controls were exposed to the TLR-3 agonist for 24, 48, or 96 hrs. (Figure 3C). The TLR-3 agonist amplified expression of several cytokines, including IL-1α, IL-1β, IL-6, and IL-8. Expression of these cytokines was higher in exposed senescent cells than cells not exposed to the TLR-3 agonist. Remarkably, the increase in SASP factor expression appeared to be greater after TLR-3 agonist exposure of senescent than non-senescent cells, suggesting that senescent cells have an amplified inflammatory response to RNA-viral PAMPs. Together with the above data showing that a TLR-3 antagonist prevents amplification of the SASP by pseudovirus, these experiments with the TLR-3 agonist indicate that signaling through TLR-3 is both necessary and sufficient for SARS-CoV-2 amplification of the SASP.

Genuine SARS-CoV-2 amplifies the SASP of senescent preadipocytes cells *via* TLR-3

To test if the effects of the pseudovirus reflect the impact of genuine SARS-CoV-2, senescent human preadipocytes were exposed to SARS-CoV-2 for 48 hrs. SARS-CoV-2 did not infect or productively replicate in preadipocytes (Figure 4A). In line with results using pseudovirus or TLR-3 agonists, senescent cells exposed to genuine SARS-CoV-2 virus had an amplified SASP response, characterized by increased IL-1α, IL-1β, IL-6, IL-8, and GMCSF mRNA levels (Figure 4B). Silencing of TLR-3 expression prevented induction of these cytokines, while silencing of TLR-4 had little if any attenuating effect (Figure 4B and Supplementary Figure 1). These results indicate that genuine SARS-CoV-2 external to senescent preadipocytes is sensed in a TLR-3-dependent manner, further exacerbating their pro-inflammatory SASP.

Lung $p16^{INK4a+}$ senescent cell burden is greater in patients dying from acute SARS-CoV-2 than from other causes

To investigate if SARS-CoV-2 infection is associated with induction of senescence, we compared $p16^{INK4a}$ expression in lungs from 10 patients who had died from SARS-CoV-2 (age 76 ±13 years; mean ± SD; 3 females, 7 males) to 6 controls (age 78 ± 19.5 years; 2 females, 4 males) who did not have COPD, asthma, or other pulmonary diseases (Mayo Clinic Tissue Registry; Mayo Clinic IRB #21-001392) as shown in Supplementary Tables 1 and 2. We found increased numbers of $p16^{INK4a}$ positive cells in the lungs of patients who had died from SARS-CoV-2 (Figure 5), consistent with the possibility that SARS-CoV-2 infection can induce cellular senescence.

DISCUSSION

We previously reported that SARS-CoV-2 surface antigen Spike-1 protein (S1), which signals through ACE2 receptors, can cause amplification of the tissue-destructive, pro-inflammatory SASP of already senescent human cells [3]. Here, we show that SARS-CoV-2 Spike pseudotyped VSV can cause non-senescent cells to become senescent through TLR-3 in kidney endothelial and lung epithelial cells. However, in future studies other cells types also need to be assessed. Additionally, the pseudovirus and genuine SARS-CoV-2 amplified the SASP through TLR-3, with senescent cells becoming more pro-inflammatory than non-senescent cells after viral exposure. Expression of TLR-3 mRNA and protein was higher in senescent than non-senescent cells. Genuine SARS-CoV-2 did not replicate to detectable levels in either senescent preadipocytes or non-senescent preadipocytes, yet the virus amplified the SASP in the former. This is consistent with the increased abundance of TLR-3 receptors on senescent *vs.* non-senescent cells contributing to SASP amplification. Furthermore, TLR-3 antagonists or depletion prevented senescence induction as well as SASP amplification by the pseudovirus and genuine SARS-CoV-2, further indicating a role for TLR-3. A TLR-3 activator amplified the SASP, paralleling effects of viral exposure, suggesting that TLR-3 signaling is both necessary and sufficient for SASP amplification by SARS-CoV-2. This exacerbation of the SASP by SARS-CoV-2 through TLR-3 might further contribute to the SASP induction by S1 that we reported previously in multiple cells to test further the generalizability of the mechanisms we reported [3]. Key findings with the pseudovirus were recapitulated with genuine SARS-CoV-2. Also, more highly $p16^{INK4a}$-expressing cells were present in lungs of patients who had died from SARS-CoV-2 than patients dying from other causes, indicating an increased senescent cell burden in SARS-CoV-2 patients. However, more senescent markers, *e.g.*, $p21^{CIP1}$, will need to be examined in patients' lungs, since $p16^{INK4a}$ expression can also be increased in non-senescent cells, such as activated macrophages [40]. Formation of new senescent cells, coupled with SASP amplification, could contribute to the markedly greater

morbidity and mortality from SARS-CoV-2 infection in patients with high pre-existing senescent cell burden than young, previously healthy patients. This includes elderly SARS-CoV-2 patients as well as younger patients with cellular senescence-linked conditions, such as obesity, diabetes, chronic respiratory, cardio-vascular, and renal diseases, cancers, or a history of chemotherapeutic or radiation treatment, among others [5, 24, 25, 31, 41].

It has been appreciated for some time that the SASP can be attenuated by "senomorphic" agents, such as rapamycin or metformin [42]. However, it has only recently become apparent that the SASP can be amplified

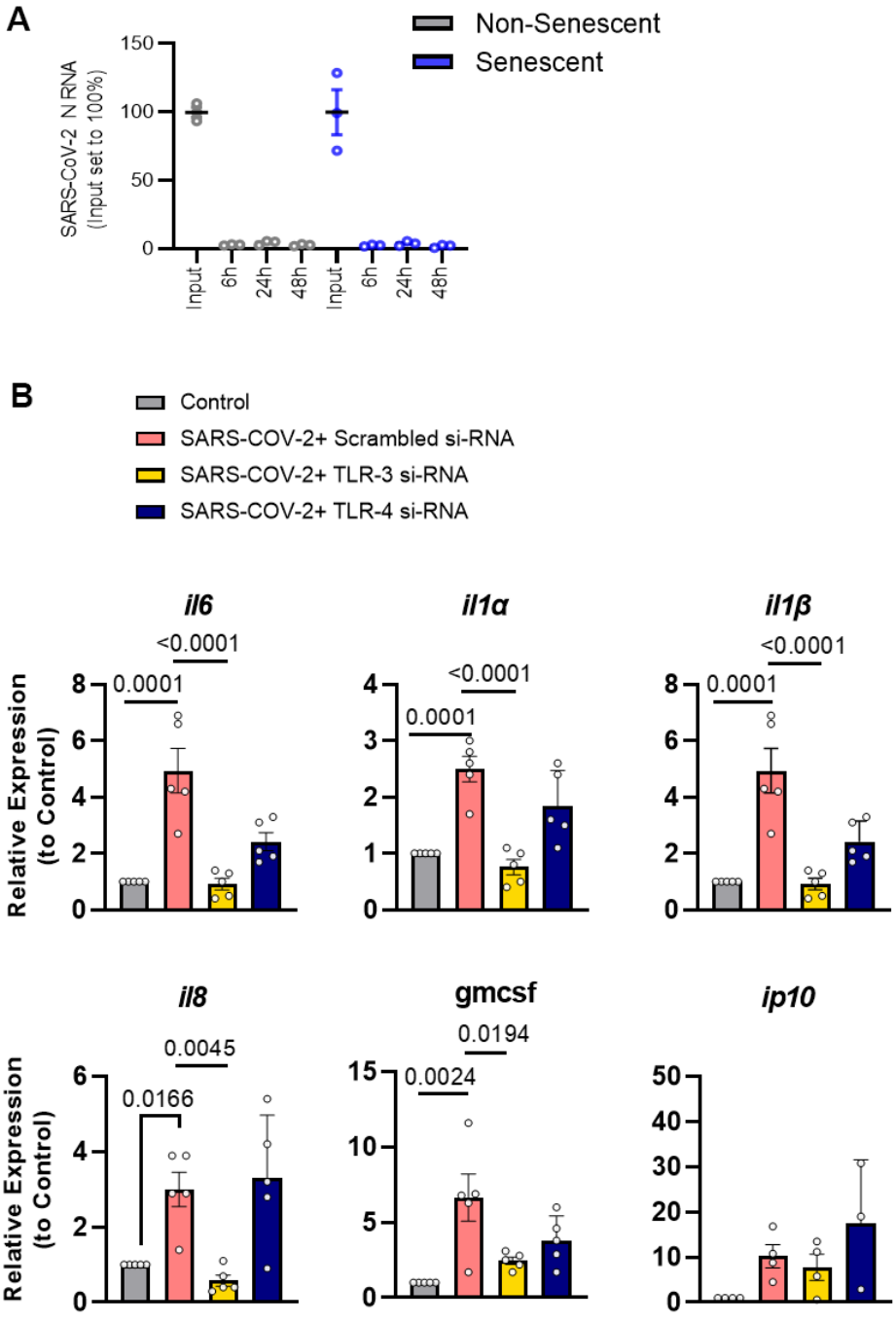

Figure 4. SARS-CoV-2 amplifies the SASP of senescent preadipocytes without infecting them. (**A**) Senescent and non-senescent preadipocytes were exposed to SARS-CoV-2 for the indicated times and assayed for infection by qPCR. (**B**) Senescent preadipocytes were treated with SARS-CoV-2 and the SASP was assayed 82 hrs. later by qPCR. Data are expressed as a function of untreated senescent cells; mean +/- SEM, repeated 1-way ANOVA and *post hoc* comparison pairwise Tukey's HSD. All other significant p values are listed in Supplementary Table 1.

by signals like PAMPs, such as lipopolysaccharide or S1 antigen, as predicted by our SASP "Amplifier/ Rheostat Hypothesis" [3]. The finding that SARS-CoV-2 exacerbates the SASP through TLR-3 is consistent with this hypothesis, which may partly explain the increased morbidity and mortality due to hyper-inflammation and tissue destruction in older and chronically-ill individuals compared to previously healthy, younger SARS-CoV-2-infected individuals. Also, consistent with this hypothesis, we recently reported that reducing senescent cell abundance genetically or pharmacologically (with senolytic agents) in old mice infected with a β-coronavirus related to SARS-CoV-2, mouse hepatitis virus (MHV), reduces their higher risk for cytokine storm and mortality than young mice [3]. Potentially, the Amplifier/Rheostat Hypothesis accounts for the increased morbidity and mortality in patients with increased pre-existing senescent cell burden from other types of infections, although further testing is needed.

Interactions between senescent cells, their SASP, and immune cells likely contribute to the impact of senescent cells on SARS-CoV-2 morbidity and mortality [14]. Immune cells can act to increase senescent cell abundance. Activated neutrophils, which accumulate extensively in lungs of severely ill SARS-CoV-2 patients [43], have the capacity to induce non-senescent cells to become senescent [10], potentially adding to the senescent cell burden caused by SARS-CoV-2 through TLR-3 dependent signalling.

Senescent cells are resistant to apoptosis [13] and are mainly removed by the immune system [14]. We previously found that the SASP causes spread of senescence to normal cells, not only locally but also at a distance [20]. These observations led us to propose the "Threshold Theory of Senescent Cell Burden". This theory, if true, suggests that above a threshold abundance, senescent cells persist and even increase in number because the rate of formation of new senescent cells exceeds senescent cell removal by the immune system. Reaching this threshold would depend on the sum of pre-existing and newly formed senescent cells. To confirm whether such a threshold model would be mathematically tenable given available data and information remains to be evaluated. New senescent cells induced by viral RNA, on top of senescent cell spread from SASP amplification due to virally-induced TLR-3 activation, S1-induced ACE2 stimulation, and increased ROS, could result in surpassing the senescent cell threshold, leading to feed-forward increases in senescent cell burden and resulting morbidity and mortality. Adding to the above, a high burden of senescent cells can impair normal immune system function [14].

A persisting or increasing burden of senescent cells, the SASP, and/or SASP amplification could combine to contribute to both immediate and long-term morbidity due to current or previous SARS-CoV-2 viremia. Consistent with this possibility, transplanting even a small number of senescent cells causes complications resembling those of coronavirus infection in mice, including frailty, weakness, decreased activity, and increased mortality [20]. Hence, increased abundance of senescent cells might contribute to the brain fog/anxiety, physical inactivity/lethargy/muscle weakness/frailty, lung fibrosis/dyspnea, and arthritis/generalized pain symptoms that can persist after acute SARS-CoV-2 infection, the so called post-acute sequelae of COVID-19. Senescent cell burden may even account, in part, for the frailty/ accelerated aging-like state that progresses four times faster in older nursing home residents *vs*. uninfected

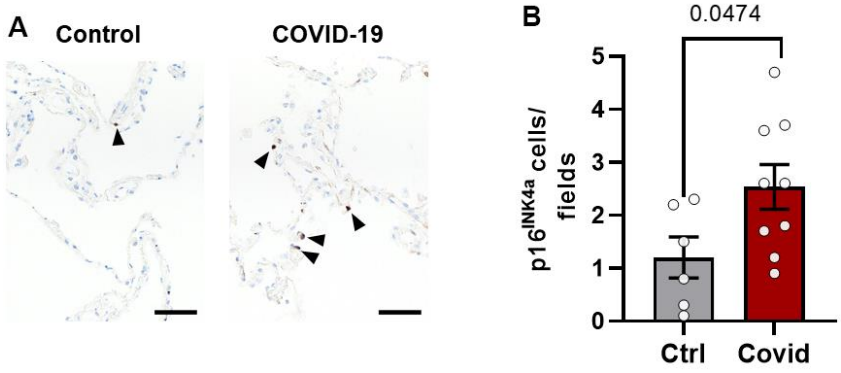

Figure 5. Lung p16INK4a+ senescent cell burden is greater in patients dying from acute SARS-CoV-2 than other causes. Lung tissue from patients who died from SARS-CoV-2 (n=9) were compared to controls (n=6) who died from other causes without lung disease (see Supplementary Tables 2, 3). (**A**) Paraffin-embedded lung autopsy tissue was sectioned and stained for p16INK4a by immunohistochemistry (black arrowheads). (**B**) Fifteen fields of alveolar tissue were randomly selected and counted. Mean +/- SEM, unpaired 2-tailed Student's t-test.

residents for months after SARS-CoV-2 viremia has resolved [44]. Based on these considerations, a clinical study has commenced to test if senescent cell burden is increased in patients with PASC *vs*. age-matched controls (Cellular Senescence and COVID-19 Long-Hauler Syndrome; clinicaltrials.gov identifier NCT04903132).

The effects of SARS-CoV-2 on SASP activation and induction of cellular senescence considered here raise a number of questions that indicate directions for further research, particularly regarding potential clinical interventions to delay, prevent, and treat short- and long-term complications of SARS-CoV-2 viremia. One such class of drugs is senolytics, agents that selectively eliminate senescent cells. Senolytics transiently disable the senescent cell anti-apoptotic pathway (SCAP) network that shields senescent cells from their own SASP and that allows them to survive despite their killing cells around them and causing tissue damage [45–50]. We recently reported that morbidity and mortality in old mice infected with ß-coronavirus, MHV, are attenuated by the senolytics, Fisetin and the combination of Dasatinib and Quercetin [3]. Clinical trials of senolytics for SARS-CoV-2 are already underway, including a SARS-CoV-2 acute hospital trial (COVID-FISETIN: Pilot in SARS-CoV-2 of Fisetin to Alleviate Dysfunction and Inflammation; NCT04476953), a skilled nursing facility trial (COVID-FIS, A Study of Fisetin for Skilled Nursing Facility Residents with COVID-19; NCT04537299), and an outpatient trial (COVFIS-HOME: COVID-19 Pilot Study of Fisetin to Alleviate Dysfunction and Disease Complications; NCT04771611). Senomorphics, such as metformin or agents related to rapamycin, such as sirolimus, offer an alternative way to attenuate the SASP [42]. Trials with senomorphics are also underway (*e.g*., metformin: NCT04510194 and NCT04727424; sirolimus: NCT04341675 and NCT 04948203). Yet another option might be to conduct trials with TLR-3 antagonists, which have been used in pre-clinical studies of SARS-CoV-2 infection [51]. These agents, which have been in clinical trials as immuno-modulators and adjuvants to enhance vaccine effectiveness, have not for the most part advanced past early phase trials [52]. While TLR-3 antagonists may prove to be of use for treating SARS-CoV-2, this might only be during a narrow window early in the course of infection because, based on the findings reported here, these agents would mainly be effective during active viremia before senescent cell abundance has been increased.

MATERIALS AND METHODS

Cell culture

Preadipocytes were isolated from abdominal subcutaneous fat biopsies obtained from subjects undergoing gastric bypass surgery. All subjects gave informed consent. The protocol was approved by the Mayo Clinic Institutional Review Board for Human Research. Cells were isolated, cultured, and were made senescent as previously described [53] and corresponding non-senescent were used as controls whenever required. Human primary renal glomerular endothelial cells (Science Cell, Cat #4000, Carlsbad, CA. USA) and human small airway epithelial cells (Cat# CC-2547, Lonza) were purchased and cultured following the manufacturer's instructions. Human preadipocytes and kidney endothelial cells were made senescent by 20 Gy irradiation and experiments were performed after 30 days and 21 days, respectively.

Reagents

Cells were treated with following reagents as indicated in the figures: 1) polyinosine-polycytidylic acid (Cat #tlrl-pic-5, Invivogen, San Diego, CA, USA), 2) (R)-2-(3-chloro-6-fluorobenzo[b]thiophene-2-carboxamido)-3-phenylpropanoic acid, Toll-Like Receptor-3/double strand RNA complex inhibitor (Cat# 614310-10MG, Burlington, MA, USA), and 3) pseudovirus (Cat #B2000052, Brainvta, Wuhan, China).

RNA extraction and rtPCR

For most studies, RNA isolation and rtPCR were performed using Trizol as in [54, 55]. Cells were washed with PBS, then Trizol and chloroform were added to each sample. Samples were centrifuged to separate the aqueous layer. RNA was purified using columns (Qiagen Kit Cat#74104) according to the manufacturer's instructions. Concentration and purity of samples were assayed using a Nanodrop spectrophotometer. Each cDNA sample was generated by reverse transcription using 1-2000 ng RNA following the manufacturer's recommended protocol (High-capacity cDNA Reverse Transcription Kit; Cat #4368813, Thermo Fisher Scientific, Waltham, MA, USA). A standard reverse transcription program was used (10 min. at 25° C, 120 min. at 37° C, 5 min. at 85° C, held at 4° C). TBP was used as a control for gene expression analysis. For the experiments related to coronavirus, we used following method for rtPCR. SARS-CoV-2 nucleoprotein (N) RNA levels were assayed in supernatants of infected samples 48 hrs. post-infection. Forward (HKU-NF): 5'-TAA TCA GAC AAG GAA CTG ATT A-3' and reverse primers (HKU-NR): 5'-CGA AGG TGT GAC TTC CAT G-3'; Probe (HKU-NP): 5'-FAM-GCA AAT TGT GCA ATT TGC GG-3'TAMRA) were from Biomers (Ulm, Germany). IL-6, IL-8, IP-10, CSF2, IL-1α, IL-1β, $p16^{INK4a}$, and $p21^{CIP1}$ primers and probes (TaqMan) were from Thermo Fisher Scientific. RNA levels were determined in cells collected from SARS-

CoV-2-infected samples 82 hrs. post-infection. Total RNA was isolated from cells or supernatants using a Viral RNA Mini Kit (#52904, Qiagen, Hilden, Germany) according to the manufacturer's instructions. rtPCR was performed according to the manufacturer's instructions using TaqMan Fast Virus 1-Step Master Mix (Cat#4444436, Thermo Fisher Scientific) and a OneStepPlus Real-Time PCR System (96-well format, fast mode). Synthetic SARS-CoV-2-RNA (Cat#Q-87194, Twist Bioscience, South San Francisco, CA, USA) was used as a quantitative standard to determine viral copy numbers. All reactions were run in duplicate. mRNAs were expressed as a function of GAPDH primer/probe sets (Cat#4310884E, Thermo Fisher Scientific). Data were analyzed by the ΔΔCt method.

Primers used are listed below.

Gene name	Primers
TLR1	Hs00413978_m1
TLR2	Hs02621280_s1
TLR3	Hs01551079_g1
TLR4	Hs00152939_m1
TLR5	Hs01920773_s1
TLR6	Hs01039989_s1
TLR7	Hs01933259_s1
TLR8	Hs07292888_s1
TLR9	Hs00370913_s1
TLR10	Hs01935337_s1
TBP	Hs00427620_m1
P16	Hs00923894_m1
P21	Hs00355782_m1
IL-1α	Hs00174092_m1
IL-1β	Hs01555410_m1
Il-6	Hs00174131_m1
IL-8	Hs00174103_m1
IP-10	Hs00171042_m1
CSF2	Hs00929873_m1

siRNA knockdown

Cells were transfected with the indicated siRNAs using RNAi max reagent (Cat#13778075; Thermo Fisher Scientific) as in [55]. Briefly, cells were transfected in antibiotic-free media in 6 well plates. 9μl RNAi max/well were mixed in 150μl OPTI-mem media and 6μl 10μM siRNA in 150μl of OPTI-MEM medium in a separate Eppendorf tube. The two tubes were mixed and incubated for 5 mins. 250μl of the mixture were added to wells. The following siRNAs were purchased from Thermo Fisher Scientific): TLR-3 siRNA (Assay ID: 107054) and TLR-4 siRNA (Assay ID: s-14195).

Western blots

Cells or tissues were homogenized in RIPA buffer (Cat #89900, Thermo Fisher Scientific) with protease inhibitors (Cat# 78430, Thermo Fisher Scientific). Proteins were loaded on SDS-PAGE gels and transferred to immuno-blot PVDF membranes (Biorad, Hercules; CA, USA). ECL Western Blotting Substrate (Pierce; cat #32106, Rockford IL, USA) was used to develop signals. TLR-3 (catalog #6961) and α-tubulin (catalog #2144) antibodies were purchased from Cell Signaling. Data were quantified using the optical densities of the specified proteins as a function of α-tubulin.

SARS-CoV-2 stock production

BetaCoV/France/IDF0372/2020 was propagated on Vero E6 infected at a multiplicity of infection (MOI) of 0.003 in serum-free medium containing 1μg/ml trypsin, as previously described [56]. Briefly, the cells were inoculated for 2 hrs. at 37° C before the inoculum was removed. The supernatant was harvested 48 hrs. post-infection upon visible cytopathic effect. To remove debris, the supernatants were centrifuged for 5 min. at 1,000 x g, then aliquoted and stored at −80° C.

Plaque-forming unit assays

Plaque-forming unit (PFU) assays were performed as previously described [57]. Briefly, SARS-CoV-2 stocks were serially diluted and confluent monolayers of Vero E6 cells infected. After incubation for 2 hrs. at 37° C with shaking every 20 min., the cells were overlaid with 1.5 ml 0.8% Avicel RC-581 (FMC/DuPont; Wilmington, DE, USA) in medium and incubated for 3 days. Cells were fixed with 4% PFA at room temperature for 45 min. After cells had been washed with PBS once, they were incubated in 0.5ml staining solution (0.5 % crystal violet and 0.1 % triton in water) at room temperature. After 20 min., the staining solution was rinsed off with water, virus-induced plaque formation quantified, and PFU/ml calculated.

Multiplex ELISA

CM was filtered and cytokine and chemokine protein levels in CM were measured using Luminex xMAP technology as in [3]. Multiplexing analysis was performed using a Luminex 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta, Canada). Data are represented as pg/ml for each SASP factor as a function of cellular density.

Human lung studies

Lungs from 9 patients who had died with SARS-CoV-2 (age 74 ±12 years; mean ± SD; 3 females, 6 males) were compared to 6 controls (age 78 ± 19 years; 2 females, 4 males) who did not have COPD, asthma, or other

pulmonary diseases (Mayo Clinic Tissue Registry; Mayo Clinic IRB #21-001392). Paraffin-embedded lung tissue was sectioned into 4µm sections, stained immunohistochemically for $p16^{INK4a+}$ cells (Clone E6H4, #705-4793, Roche Tissue Diagnostics, Indianapolis, IN, USA) using a VENTANA Discovery ULTRA instrument (Ventana Medical Systems; Oro Valley, AZ, USA), counterstained with hematoxylin, and scanned using a 40x objective Motic Slide Scanner (Motic Company; Xiamen, China). Images were virtually sliced into 300 x 400 µm numbered fields that were selected using a Microsoft Excel random number generator. Fifteen fields of $p16^{INK4a+}$ lung cells were so counted/subject. Results were analyzed by an unpaired 2-tailed t-test.

Statistical analysis

All figures were plotted using Prism 9.0 (GraphPad). P value ≤ 0.05 (two-sided) was considered statistically significant. Student's t-test was used to compare the equality of means from two independent samples, and Welch's correction was performed when two samples were determined to have significantly unequal variances (Levene's test). One-way ANOVA was used to compare means from three or more samples, and two-way ANOVA was used when there were two predictors. Tukey's Honestly Significant Difference (HSD) was used for *post hoc* pairwise comparisons where 4 means were being compared, while Fisher's Least Significant Difference (LSD) procedure was used when comparing 3 means. Paired t-tests and repeated measures ANOVA were used to account for nesting, *i.e*. correlated data across cells (preadipocytes) from the same subject. All p values (<0.05) are indicated in the figures and Supplementary Table 1.

AUTHOR CONTRIBUTIONS

J.L.K. and T.T. generated the overall concept of the study. U.T., R.N., L.L., Y.Z., N.G., T.P., J.M.E.N., and A.X. performed experiments in this study. D.A.B., E.P., and S.L.D performed the statistical analyses. U.T, E.O.W.G., S.S., P.D.R, N.J.L., F.K., K.M.J.S., T.T., and J.L.K. designed the study and wrote the manuscript. All authors read, edited, and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

Patents on senolytic drugs and their uses are held by Mayo Clinic and the University of Minnesota. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic Conflicts of interest policies.

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REFERENCES

1. Finelli L, Gupta V, Petigara T, Yu K, Bauer KA, Puzniak LA. Mortality Among US Patients Hospitalized With SARS-CoV-2 Infection in 2020. JAMA Netw Open. 2021; 4:e216556. https://doi.org/10.1001/jamanetworkopen.2021.6556 PMID:33830226

2. Ganesh R, Grach SL, Bierle DM, Salonen BR, Collins NM, Joshi AY, Boeder N, Anstine CV, Mueller MR, Wight EC, Croghan IT, Badley AD, Carter RE, et al. The Female Predominant Persistent Immune Dysregulation of the Post COVID Syndrome: A Cohort Study. medRxiv. 2021. https://doi.org/10.1101/2021.05.25.21257820

3. Camell CD, Yousefzadeh MJ, Zhu Y, Prata LG, Huggins MA, Pierson M, Zhang L, O'Kelly RD, Pirtskhalava T, Xun P, Ejima K, Xue A, Tripathi U, et al. Senolytics reduce coronavirus-related mortality in old mice. Science. 2021; 373:eabe4832. https://doi.org/10.1126/science.abe4832 PMID:34103349

4. Song S, Lam EW, Tchkonia T, Kirkland JL, Sun Y. Senescent Cells: Emerging Targets for Human Aging and Age-Related Diseases. Trends Biochem Sci. 2020; 45:578–92. https://doi.org/10.1016/j.tibs.2020.03.008 PMID:32531228

5. Khosla S, Farr JN, Tchkonia T, Kirkland JL. The role of cellular senescence in ageing and endocrine disease. Nat Rev Endocrinol. 2020; 16:263–75. https://doi.org/10.1038/s41574-020-0335-y PMID:32161396

6. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007; 8:729–40.

https://doi.org/10.1038/nrm2233
PMID:17667954

7. Anderson R, Lagnado A, Maggiorani D, Walaszczyk A, Dookun E, Chapman J, Birch J, Salmonowicz H, Ogrodnik M, Jurk D, Proctor C, Correia-Melo C, Victorelli S, et al. Length-independent telomere damage drives post-mitotic cardiomyocyte senescence. EMBO J. 2019; 38:e100492. https://doi.org/10.15252/embj.2018100492 PMID:30737259

8. Conley SM, Hickson LJ, Kellogg TA, McKenzie T, Heimbach JK, Taner T, Tang H, Jordan KL, Saadiq IM, Woollard JR, Isik B, Afarideh M, Tchkonia T, et al. Human Obesity Induces Dysfunction and Early Senescence in Adipose Tissue-Derived Mesenchymal Stromal/Stem Cells. Front Cell Dev Biol. 2020; 8:197. https://doi.org/10.3389/fcell.2020.00197 PMID:32274385

9. Parikh P, Britt RD Jr, Manlove LJ, Wicher SA, Roesler A, Ravix J, Teske J, Thompson MA, Sieck GC, Kirkland JL, LeBrasseur N, Tschumperlin DJ, Pabelick CM, Prakash YS. Hyperoxia-induced Cellular Senescence in Fetal Airway Smooth Muscle Cells. Am J Respir Cell Mol Biol. 2019; 61:51–60. https://doi.org/10.1165/rcmb.2018-0176OC PMID:30508396

10. Lagnado A, Leslie J, Ruchaud-Sparagano MH, Victorelli S, Hirsova P, Ogrodnik M, Collins AL, Vizioli MG, Habiballa L, Saretzki G, Evans SA, Salmonowicz H, Hruby A, et al. Neutrophils induce paracrine telomere dysfunction and senescence in ROS-dependent manner. EMBO J. 2021; 40:e106048. https://doi.org/10.15252/embj.2020106048 PMID:33764576

11. Pathak RU, Soujanya M, Mishra RK. Deterioration of nuclear morphology and architecture: A hallmark of senescence and aging. Ageing Res Rev. 2021; 67:101264. https://doi.org/10.1016/j.arr.2021.101264 PMID:33540043

12. González-Gualda E, Baker AG, Fruk L, Muñoz-Espín D. A guide to assessing cellular senescence *in vitro* and *in vivo*. FEBS J. 2021; 288:56–80. https://doi.org/10.1111/febs.15570 PMID:32961620

13. Wang E. Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Cancer Res. 1995; 55:2284–92. PMID:7757977

14. Prata LG, Ovsyannikova IG, Tchkonia T, Kirkland JL. Senescent cell clearance by the immune system: Emerging therapeutic opportunities. Semin Immunol. 2018; 40:101275. https://doi.org/10.1016/j.smim.2019.04.003 PMID:31088710

15. Kale A, Sharma A, Stolzing A, Desprez PY, Campisi J. Role of immune cells in the removal of deleterious senescent cells. Immun Ageing. 2020; 17:16. https://doi.org/10.1186/s12979-020-00187-9 PMID:32518575

16. Kandhaya-Pillai R, Miro-Mur F, Alijotas-Reig J, Tchkonia T, Kirkland JL, Schwartz S. TNFα-senescence initiates a STAT-dependent positive feedback loop, leading to a sustained interferon signature, DNA damage, and cytokine secretion. Aging (Albany NY). 2017; 9:2411–35. https://doi.org/10.18632/aging.101328 PMID:29176033

17. Zhu Y, Armstrong JL, Tchkonia T, Kirkland JL. Cellular senescence and the senescent secretory phenotype in age-related chronic diseases. Curr Opin Clin Nutr Metab Care. 2014; 17:324–28. https://doi.org/10.1097/MCO.0000000000000065 PMID:24848532

18. Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest. 2013; 123:966–72. https://doi.org/10.1172/JCI64098 PMID:23454759

19. Coppé JP, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol. 2008; 6:2853–68. https://doi.org/10.1371/journal.pbio.0060301 PMID:19053174

20. Xu M, Pirtskhalava T, Farr JN, Weigand BM, Palmer AK, Weivoda MM, Inman CL, Ogrodnik MB, Hachfeld CM, Fraser DG, Onken JL, Johnson KO, Verzosa GC, et al. Senolytics improve physical function and increase lifespan in old age. Nat Med. 2018; 24:1246–56. https://doi.org/10.1038/s41591-018-0092-9 PMID:29988130

21. Iske J, Seyda M, Heinbokel T, Maenosono R, Minami K, Nian Y, Quante M, Falk CS, Azuma H, Martin F, Passos JF, Niemann CU, Tchkonia T, et al. Senolytics prevent mt-DNA-induced inflammation and promote the survival of aged organs following transplantation. Nat Commun. 2020; 11:4289. https://doi.org/10.1038/s41467-020-18039-x PMID:32855397

22. Tchkonia T, Palmer AK, Kirkland JL. New Horizons: Novel Approaches to Enhance Healthspan Through Targeting Cellular Senescence and Related Aging

Mechanisms. J Clin Endocrinol Metab. 2021; 106:e1481–87.
https://doi.org/10.1210/clinem/dgaa728
PMID:33155651

23. Wissler Gerdes EO, Zhu Y, Weigand BM, Tripathi U, Burns TC, Tchkonia T, Kirkland JL. Cellular senescence in aging and age-related diseases: Implications for neurodegenerative diseases. Int Rev Neurobiol. 2020; 155:203–34.
https://doi.org/10.1016/bs.irn.2020.03.019
PMID:32854855

24. Wyld L, Bellantuono I, Tchkonia T, Morgan J, Turner O, Foss F, George J, Danson S, Kirkland JL. Senescence and Cancer: A Review of Clinical Implications of Senescence and Senotherapies. Cancers (Basel). 2020; 12:2134.
https://doi.org/10.3390/cancers12082134
PMID:32752135

25. Pignolo RJ, Passos JF, Khosla S, Tchkonia T, Kirkland JL. Reducing Senescent Cell Burden in Aging and Disease. Trends Mol Med. 2020; 26:630–38.
https://doi.org/10.1016/j.molmed.2020.03.005
PMID:32589933

26. Kim SR, Jiang K, Ferguson CM, Tang H, Chen X, Zhu X, Hickson LJ, Tchkonia T, Kirkland JL, Lerman LO. Transplanted senescent renal scattered tubular-like cells induce injury in the mouse kidney. Am J Physiol Renal Physiol. 2020; 318:F1167–76.
https://doi.org/10.1152/ajprenal.00535.2019
PMID:32223312

27. Palmer AK, Gustafson B, Kirkland JL, Smith U. Cellular senescence: at the nexus between ageing and diabetes. Diabetologia. 2019; 62:1835–41.
https://doi.org/10.1007/s00125-019-4934-x
PMID:31451866

28. Farr JN, Rowsey JL, Eckhardt BA, Thicke BS, Fraser DG, Tchkonia T, Kirkland JL, Monroe DG, Khosla S. Independent Roles of Estrogen Deficiency and Cellular Senescence in the Pathogenesis of Osteoporosis: Evidence in Young Adult Mice and Older Humans. J Bone Miner Res. 2019; 34:1407–18.
https://doi.org/10.1002/jbmr.3729 PMID:30913313

29. Lewis-McDougall FC, Ruchaya PJ, Domenjo-Vila E, Shin Teoh T, Prata L, Cottle BJ, Clark JE, Punjabi PP, Awad W, Torella D, Tchkonia T, Kirkland JL, Ellison-Hughes GM. Aged-senescent cells contribute to impaired heart regeneration. Aging Cell. 2019; 18:e12931.
https://doi.org/10.1111/acel.12931
PMID:30854802

30. Ogrodnik M, Zhu Y, Langhi LGP, Tchkonia T, Krüger P, Fielder E, Victorelli S, Ruswhandi RA, Giorgadze N, Pirtskhalava T, Podgorni O, Enikolopov G, Johnson KO, et al. Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. Cell Metab. 2019; 29:1061–77.e8.
https://doi.org/10.1016/j.cmet.2018.12.008
PMID:30612898

31. Tchkonia T, Kirkland JL. Aging, Cell Senescence, and Chronic Disease: Emerging Therapeutic Strategies. JAMA. 2018; 320:1319–20.
https://doi.org/10.1001/jama.2018.12440
PMID:30242336

32. Ogrodnik M, Evans SA, Fielder E, Victorelli S, Kruger P, Salmonowicz H, Weigand BM, Patel AD, Pirtskhalava T, Inman CL, Johnson KO, Dickinson SL, Rocha A, et al. Whole-body senescent cell clearance alleviates age-related brain inflammation and cognitive impairment in mice. Aging Cell. 2021; 20:e13296.
https://doi.org/10.1111/acel.13296 PMID:33470505

33. Bian X, Griffin TP, Zhu X, Islam MN, Conley SM, Eirin A, Tang H, O'Shea PM, Palmer AK, McCoy RG, Herrmann SM, Mehta RA, Woollard JR, et al. Senescence marker activin A is increased in human diabetic kidney disease: association with kidney function and potential implications for therapy. BMJ Open Diabetes Res Care. 2019; 7:e000720.
https://doi.org/10.1136/bmjdrc-2019-000720
PMID:31908790

34. Schafer MJ, White TA, Iijima K, Haak AJ, Ligresti G, Atkinson EJ, Oberg AL, Birch J, Salmonowicz H, Zhu Y, Mazula DL, Brooks RW, Fuhrmann-Stroissnigg H, et al. Cellular senescence mediates fibrotic pulmonary disease. Nat Commun. 2017; 8:14532.
https://doi.org/10.1038/ncomms14532
PMID:28230051

35. Xu M, Bradley EW, Weivoda MM, Hwang SM, Pirtskhalava T, Decklever T, Curran GL, Ogrodnik M, Jurk D, Johnson KO, Lowe V, Tchkonia T, Westendorf JJ, Kirkland JL. Transplanted Senescent Cells Induce an Osteoarthritis-Like Condition in Mice. J Gerontol A Biol Sci Med Sci. 2017; 72:780–85.
https://doi.org/10.1093/gerona/glw154
PMID:27516624

36. Laing AG, Lorenc A, Del Molino Del Barrio I, Das A, Fish M, Monin L, Muñoz-Ruiz M, McKenzie DR, Hayday TS, Francos-Quijorna I, Kamdar S, Joseph M, Davies D, et al. Author Correction: A dynamic COVID-19 immune signature includes associations with poor prognosis. Nat Med. 2020; 26:1951.
https://doi.org/10.1038/s41591-020-01186-5
PMID:33247289

37. Lester SN, Li K. Toll-like receptors in antiviral innate immunity. J Mol Biol. 2014; 426:1246–64.
https://doi.org/10.1016/j.jmb.2013.11.024
PMID:24316048

38. Khanmohammadi S, Rezaei N. Role of Toll-like receptors in the pathogenesis of COVID-19. J Med Virol. 2021; 93:2735–39. https://doi.org/10.1002/jmv.26826 PMID:33506952

39. Condor Capcha JM, Lambert G, Dykxhoorn DM, Salerno AG, Hare JM, Whitt MA, Pahwa S, Jayaweera DT, Shehadeh LA. Generation of SARS-CoV-2 Spike Pseudotyped Virus for Viral Entry and Neutralization Assays: A 1-Week Protocol. Front Cardiovasc Med. 2021; 7:618651. https://doi.org/10.3389/fcvm.2020.618651 PMID:33521067

40. Hall BM, Balan V, Gleiberman AS, Strom E, Krasnov P, Virtuoso LP, Rydkina E, Vujcic S, Balan K, Gitlin II, Leonova KI, Consiglio CR, Gollnick SO, et al. p16(Ink4a) and senescence-associated β-galactosidase can be induced in macrophages as part of a reversible response to physiological stimuli. Aging (Albany NY). 2017; 9:1867–84. https://doi.org/10.18632/aging.101268 PMID:28768895

41. Palmer AK, Tchkonia T, Kirkland JL. Senolytics: Potential for Alleviating Diabetes and Its Complications. Endocrinology. 2021; 162:bqab058. https://doi.org/10.1210/endocr/bqab058 PMID:33705532

42. Robbins PD, Jurk D, Khosla S, Kirkland JL, LeBrasseur NK, Miller JD, Passos JF, Pignolo RJ, Tchkonia T, Niedernhofer LJ. Senolytic Drugs: Reducing Senescent Cell Viability to Extend Health Span. Annu Rev Pharmacol Toxicol. 2021; 61:779–803. https://doi.org/10.1146/annurev-pharmtox-050120-105018 PMID:32997601

43. Cavalcante-Silva LH, Carvalho DC, Lima ÉA, Galvão JG, da Silva JS, Sales-Neto JM, Rodrigues-Mascarenhas S. Neutrophils and COVID-19: The road so far. Int Immunopharmacol. 2021; 90:107233. https://doi.org/10.1016/j.intimp.2020.107233 PMID:33290963

44. Greco GI, Noale M, Trevisan C, Zatti G, Dalla Pozza M, Lazzarin M, Haxhiaj L, Ramon R, Imoscopi A, Bellon S, Maggi S, Sergi G. Increase in Frailty in Nursing Home Survivors of Coronavirus Disease 2019: Comparison With Noninfected Residents. J Am Med Dir Assoc. 2021; 22:943–47.e3. https://doi.org/10.1016/j.jamda.2021.02.019 PMID:33757725

45. Wissler Gerdes EO, Zhu Y, Tchkonia T, Kirkland JL. Discovery, development, and future application of senolytics: theories and predictions. FEBS J. 2020; 287:2418–27. https://doi.org/10.1111/febs.15264 PMID:32112672

46. Zhu Y, Tchkonia T, Pirtskhalava T, Gower AC, Ding H, Giorgadze N, Palmer AK, Ikeno Y, Hubbard GB, Lenburg M, O'Hara SP, LaRusso NF, Miller JD, et al. The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell. 2015; 14:644–58. https://doi.org/10.1111/acel.12344 PMID:25754370

47. Zhu Y, Tchkonia T, Fuhrmann-Stroissnigg H, Dai HM, Ling YY, Stout MB, Pirtskhalava T, Giorgadze N, Johnson KO, Giles CB, Wren JD, Niedernhofer LJ, Robbins PD, Kirkland JL. Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. Aging Cell. 2016; 15:428–35. https://doi.org/10.1111/acel.12445 PMID:26711051

48. Zhu Y, Doornebal EJ, Pirtskhalava T, Giorgadze N, Wentworth M, Fuhrmann-Stroissnigg H, Niedernhofer LJ, Robbins PD, Tchkonia T, Kirkland JL. New agents that target senescent cells: the flavone, fisetin, and the BCL-X_L inhibitors, A1331852 and A1155463. Aging (Albany NY). 2017; 9:955–63. https://doi.org/10.18632/aging.101202 PMID:28273655

49. Fuhrmann-Stroissnigg H, Ling YY, Zhao J, McGowan SJ, Zhu Y, Brooks RW, Grassi D, Gregg SQ, Stripay JL, Dorronsoro A, Corbo L, Tang P, Bukata C, et al. Identification of HSP90 inhibitors as a novel class of senolytics. Nat Commun. 2017; 8:422. https://doi.org/10.1038/s41467-017-00314-z PMID:28871086

50. Yousefzadeh MJ, Zhu Y, McGowan SJ, Angelini L, Fuhrmann-Stroissnigg H, Xu M, Ling YY, Melos KI, Pirtskhalava T, Inman CL, McGuckian C, Wade EA, Kato JI, et al. Fisetin is a senotherapeutic that extends health and lifespan. EBioMedicine. 2018; 36:18–28. https://doi.org/10.1016/j.ebiom.2018.09.015 PMID:30279143

51. Mukherjee R, Bhattacharya A, Bojkova D, Mehdipour AR, Shin D, Khan KS, Hei-Yin Cheung H, Wong KB, Ng WL, Cinatl J, Geurink PP, van der Heden van Noort GJ, Rajalingam K, et al. Famotidine inhibits toll-like receptor 3-mediated inflammatory signaling in SARS-CoV-2 infection. J Biol Chem. 2021; 297:100925. https://doi.org/10.1016/j.jbc.2021.100925 PMID:34214498

52. Anwar MA, Shah M, Kim J, Choi S. Recent clinical trends in Toll-like receptor targeting therapeutics. Med Res Rev. 2019; 39:1053–90. https://doi.org/10.1002/med.21553 PMID:30450666

53. Tchkonia T, Tchoukalova YD, Giorgadze N, Pirtskhalava T, Karagiannides I, Forse RA, Koo A, Stevenson M, Chinnappan D, Cartwright A, Jensen MD, Kirkland JL. Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and

apoptosis varies among fat depots. Am J Physiol Endocrinol Metab. 2005; 288:E267–77. https://doi.org/10.1152/ajpendo.00265.2004 PMID:15383371

54. Palmer AK, Xu M, Zhu Y, Pirtskhalava T, Weivoda MM, Hachfeld CM, Prata LG, van Dijk TH, Verkade E, Casaclang-Verzosa G, Johnson KO, Cubro H, Doornebal EJ, et al. Targeting senescent cells alleviates obesity-induced metabolic dysfunction. Aging Cell. 2019; 18:e12950. https://doi.org/10.1111/acel.12950 PMID:30907060

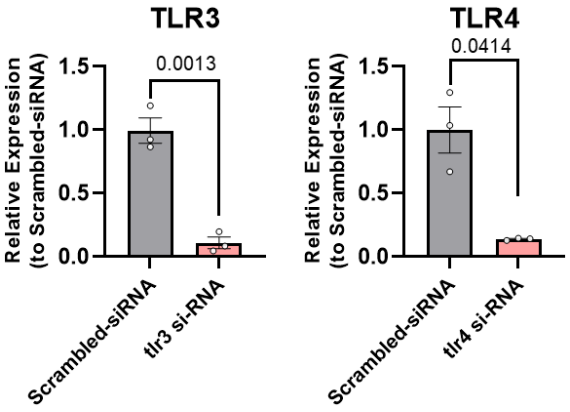
55. Xu M, Palmer AK, Ding H, Weivoda MM, Pirtskhalava T, White TA, Sepe A, Johnson KO, Stout MB, Giorgadze N, Jensen MD, LeBrasseur NK, Tchkonia T, Kirkland JL. Targeting senescent cells enhances adipogenesis and metabolic function in old age. Elife. 2015; 4:e12997. https://doi.org/10.7554/eLife.12997 PMID:26687007

56. Hayn M, Hirschenberger M, Koepke L, Nchioua R, Straub JH, Klute S, Hunszinger V, Zech F, Prelli Bozzo C, Aftab W, Christensen MH, Conzelmann C, Müller JA, et al. Systematic functional analysis of SARS-CoV-2 proteins uncovers viral innate immune antagonists and remaining vulnerabilities. Cell Rep. 2021; 35:109126. https://doi.org/10.1016/j.celrep.2021.109126 PMID:33974846

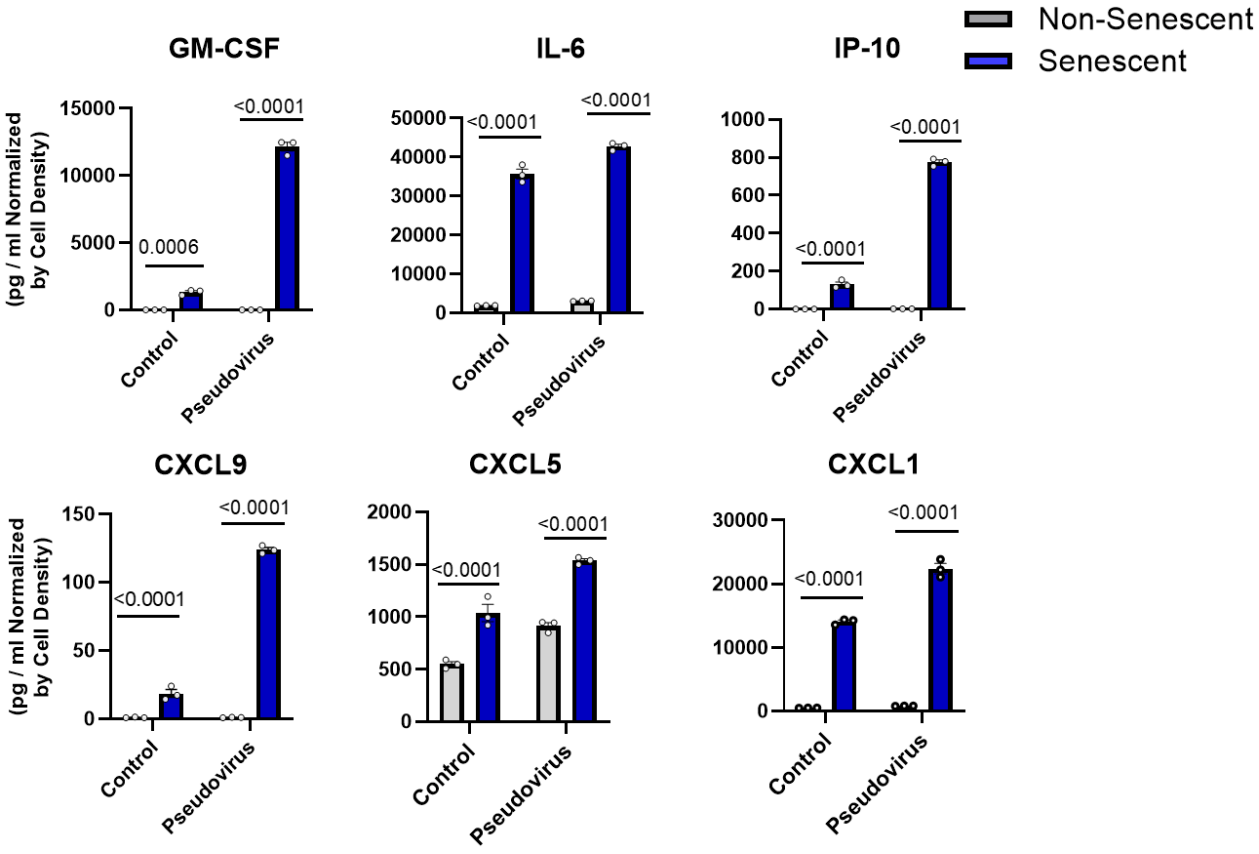
57. Conzelmann C, Gilg A, Groß R, Schütz D, Preising N, Ständker L, Jahrsdörfer B, Schrezenmeier H, Sparrer KM, Stamminger T, Stenger S, Münch J, Müller JA. An enzyme-based immunodetection assay to quantify SARS-CoV-2 infection. Antiviral Res. 2020; 181:104882. https://doi.org/10.1016/j.antiviral.2020.104882 PMID:32738255

SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. siRNA mediated knockdown of TLR-3 and TLR-4 in senescent preadipocytes. Knockdown by siRNA was confirmed by rtPCR after 2 days in senescent preadipocytes (n=3). Data are expressed as a function of scrambled siRNA-treated senescent cells; mean +/- SEM, paired 2-tailed Student's t-tests.



Supplementary Figure 2. Pseudovirus amplifies the SASP in senescent kidney endothelial cells. SASP factors were assayed in senescent and non-senescent kidney endothelial cells treated with pseudovirus for 96 hrs. Data are shown as a function of cell number; mean +/- SEM, 2-way ANOVA and *post hoc* comparison Fisher's LSD.

Supplementary Tables

Supplementary Table 1. Significant p values in addition to those shown in Figures 3, 4.

Figures	Comparisons	p values
3 B	IL-1β, TLR-3 antagonist *vs.* TLR-3 siRNA	0.0114
	IP-10, TLR-3 antagonist *vs.* TLR-3 siRNA	0.0175
3 C	Il-1α senescent, 24 *vs.* 96 hrs.	0.0466
4 B	Il-1α, TLR-4 siRNA *vs.* control	0.0132
	IL-6, TLR-3 siRNA *vs.* control	0.0450
	IL-6, TLR-4 siRNA *vs.* control	0.0193
	IL-8, TLR-4 siRNA *vs.* control	0.0065
	IP-10, TLR-4 siRNA *vs.* control	0.0288

Supplementary Table 2. Information about the control lung biopsy patients in Figure 5.

Gender	Cause of death	Age
F	Car accident	92
F	Car accident	100
M	Myocardial infarction (from atherosclerotic cardiovascular disease)	52
M	Drowning. Lungs without significant diagnostic abnormalities. No *rigor mortis* (death less than 24 hrs.)	72
M	Cerebrovascular accident	92
M	Dementia/malnutrition	59

Supplementary Table 3. Information about the COVID lung biopsy patients in Figure 5.

Gender	Cause of death/specified COVID complication	Age
M	Respiratory failure due to COVID	71
M	Developed severe hypoxemia. Bronchoscopy did not reveal clear reversible cause	73
M	SARS-CoV-2 bronchopneumonia, lethargic, generalized muscle weakness, hypotension, and continued hallucinations	93
F	Aspiration pneumonia in the setting of a recent SARS-CoV-2 infection. The deceased began antibiotic therapy, however patient experienced significant decline in mentation and renal function	89
M	Acute respiratory distress syndrome (ARDS). Family elected to have compassionate ventilator withdrawal	72
F	COVID-19 associated pneumonia in the setting of multiple chronic medical conditions and missed hemodialysis	71
F	COVID-19 infection requiring intubation, multiple strokes with residual left hemiparesis, diabetes mellitus type 2, coronary artery disease, essential hypertension, acute respiratory failure, and cognitive decline	70
M	CT chest showed findings consistent with COVID infection with profound hypoxemia. Palliative care was consulted and patient transitioned to DNR	76
M	Multiple GI bleeds. Pneumothorax infections. Massive transfusion and death	51

Research Paper

Centenarians exposed to the Spanish flu in their early life better survived to COVID-19

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ABSTRACT

Background: Although it is known that mortality due to COVID–19 increases progressively with age, the probability of dying from this serious infection among the oldest-old population is little known, and controversial data are found in literature.

Methods: We examine the mortality by year and month of birth of Belgians who had turned 100 during the current COVID-19 pandemic and whose birth fell on the years around the end the First World War and the outbreak of the H1N1 "Spanish flu" pandemic.

Findings: The COVID-19 mortality of the "older" centenarians is significantly lower than that of "younger" centenarians, and this difference between the two groups reaches a maximum on August 1, 1918 as the discriminating cut-off date of birth. Having excluded the plausible impact of the end of WWI it becomes clear that this date corresponds to the time of reporting the first victims of the Spanish flu pandemic in Belgium.

Interpretation: In this study, the striking temporal coincidence between the outbreak of the Spanish flu epidemic and the birth of the cohorts characterized by greater fragility towards COVID-19 in 2020 strongly suggests a link between exposure to 1918 H1N1 pandemic influenza and resistance towards 2020 SARS-Cov-2. It can be speculated that the lifetime persistence of cross-reactive immune mechanisms has enabled centenarians exposed to the Spanish flu to overcome the threat of COVID-19 a century later.

INTRODUCTION

Globally, the COVID-19 pandemic has become one of the most serious challenges to public health, affecting millions of lives and families [1]. With 11,500,000 people and 20,000 deaths attributed to COVID-19 in 2020, Belgium exhibited the highest mortality rate per inhabitant worldwide [2]. The first case of infection by the coronavirus was confirmed on February 2 and the first death attributed to the virus was reported on March 10. Death waves occurred twice in 2020, which peaked in April and November [3].

Among the 127,407 deaths recorded in 2020, 56,258 were of people aged 85 and above, constituting a 35.7% increase compared with the average number of deaths from 2009 to 2019, while the excess mortality was 18.3% for the whole population. A total of 1079 centenarians died in 2020 compared with an average of 826 in the period 2009–2019, representing a 30.6% increase. In 2020, 929 centenarians died starting from March 10 when the first death due to COVID-19 was recorded. When data on overall mortality among centenarians by month is compared with the deaths of 190 centenarians ascribable to COVID-19 [4], it clearly

appears that the excess mortality risk (EMR, hereafter) to centenarians is related to COVID-19 excluding some additional deaths due to the heat wave in early August.

In order to explain the disproportionate increase in the number of deaths among the oldest old, compared with that usually observed during the last decade, a more in-depth analysis is required, focusing on the size of birth cohorts and, more precisely, on the distribution, by year of birth, of the number of people alive in Belgium on March 10, 2020. Interestingly, at the end of World War I (WWI), the number of births varied largely and almost doubled starting from August 1919 (9 months after the end of WWI). Consequently, an increased number of neo-centenarians emerged in Belgium starting from the summer of 2019; 493 among the 929 centenarians who died from March 10 were between their 100th and 101st birthdays, that is, 53% compared with 39% in 2019.

Centenarians and COVID-19 pandemic

Although widely emphasized by the media [5], the resilience of centenarians to COVID-19 remains a controversial issue that is now increasingly attracting researchers. Couderc et al. [6], in a study on 321 nursing home residents in Southern France, including 12 centenarians, reported a higher mortality rate among these centenarians (50% vs 24.6%) compared with other younger residents, corresponding to one of the lowest survival rates compared with other published series [7]. Similarly, Marcon et al. [8], in a study on 42 centenarians from North-Eastern Italy as part of the CaT (Centenari a Trieste) project, observed that COVID-19-related mortality among long-lived individuals was higher than that of the population between 50 and 80 years of age, and that the mortality rate among the oldest women exceeded that of men. Overall, these data suggest that despite their ability to reach the extremes of human lifespan, centenarians are not particularly resistant to COVID-19 compared with the general population. In this context, a first research question arises: Does the oldest old, and more specifically the centenarians, die more than usually during the COVID-19 pandemic?

MATERIALS AND METHODS

The following data is used in this analysis:

- Number of deaths by single year of age at death recorded in Belgium in 2009-2020 [9] and more detailed data for the years 2016–2020 with dates of birth and death and population stock provided the Centre de Démographie (Uclouvain).
- Number of deaths of centenarians attributed to COVID-19 provided by Sciensano by year of birth, age at death, and month of death in 2020. Registered COVID-19 related deaths include confirmed and possible COVID-19 deaths. A case can be confirmed either by a chest CT scan with clinical presentation or a laboratory test. Possible deaths are those who meet the clinical criteria, whether or not there is an epidemiological link to a confirmed case [3].

In this study, mortality risk is defined as the probability of a given birth cohort of centenarians alive at the beginning of the period to die between March 10 and December 31, 2020. Notably, this is not the probability to die between two exact ages but between two exact dates.

The EMR is evaluated by comparing these observed probabilities with the expected ones. The latter are linearly extrapolated from the corresponding observed probability for the years 1991–2019 and smoothed for the oldest ages. A special attention is devoted to estimating the probability of death between March 10th and December 31st, excluding the mortality at the beginning of the year. Since the number of centenarians alive on March 10th for each single month and year of birth was small, a moving average with a bandwidth of 12 months was used for calculating these probabilities. The EMR is equivalent to the ratio between the observed and expected number of deaths for the period of the pandemic. To compute the corresponding confidence interval the usual formulas for mortality ratio is used.

In the present study two questions are addressed that are not necessarily related to each other. The first is whether the resistance of centenarians to SARS-Cov-2 infection is generally greater than that of younger individuals. Despite the strong theoretical interest of this question, the literature on this subject is scanty and does not provide sufficient elements to draw a definitive conclusion. The second and more specific question is whether the mortality of centenarians during the COVID-19 epidemic may depend on whether they were born before or after the 1918 flu pandemic. Unlike the first question, which could in principle be answered using available data, responding to the second is much more problematic, given the extremely small number of individuals exposed to both epidemics.

RESULTS

Figure 1 illustrates the EMR calculated by single year of birth during the pandemic for people born till 1935 (see detailed data in Supplementary Table 1).

The EMR by single year of age is considerably stable except for centenarians born in 1919 or earlier; the EMR suddenly drops to virtually approach unity for those born in 1915. Hence, a second research question, complementary to the previous one, can be raised: Is there a significant survival difference concerning the 2020 COVID-19 pandemic between "younger" and "older" centenarians? And if there is any difference, when did the largest difference appear, considering the exact date of birth of centenarians?

To answer these questions, we try to increase the temporal resolution of the mortality analysis by considering the probability of dying according to month of birth. Owing to the difference in the months' length and to avoid any possible seasonal effect on mortality risk, the EMR was calculated using a moving average aggregated with a group of 12 successive months.

Figure 2 illustrates the calculation of the EMR during the 2020 pandemic for those born in the years 1916-1921 by year and month of birth. Therefore, we use a 12–months moving average with 5% confidence intervals.

The greatest increase in the EMR corresponds to cohorts born between March 1918 and March 1919. To determine the point of maximum increase of EMR more accurately, we adopt a complementary method that compares the calculated EMR, as moving average, in two adjacent periods of 12 months each. The best cut point is defined as that which maximizes the difference between the two periods of 12 months, before and after that date.

In Figure 3, the confidence intervals indicate that the difference is significantly different from zero during the aforementioned period, and the best cut point maximizing that difference coincides with August 1, 1918 with a higher 29% EMR for centenarians born in the 12 months after that date (EMR = 1.474) compared with those born in the 12 months before (EMR = 1.188).

DISCUSSION

In this paper, using statistics on the oldest old population living in Belgium, we report that centenarians born before August 1, 1918, globally display a lower EMR during the 2020 COVID-19

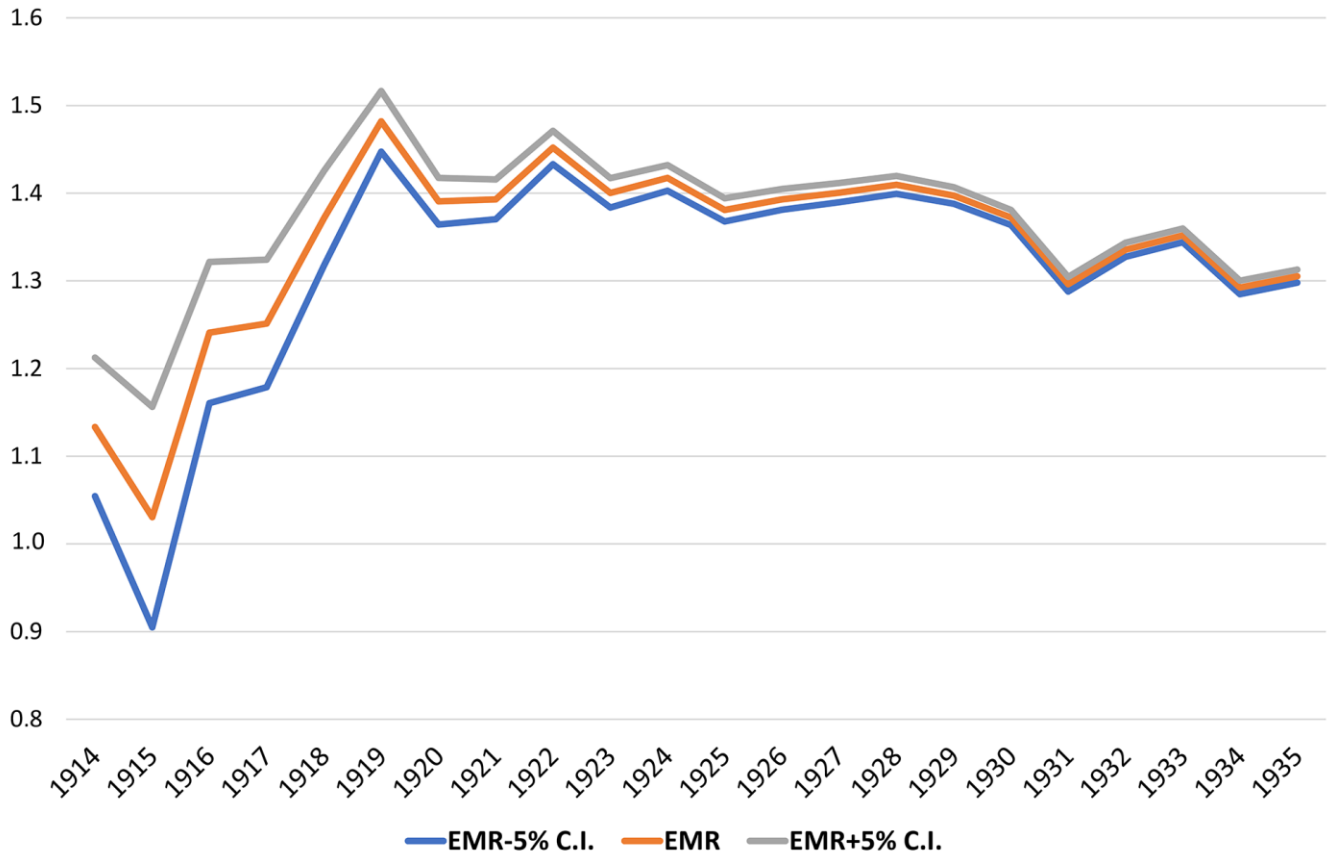

Figure 1. EMR during the pandemic by single year of birth from 1914 to 1935 with a 5% confidence interval.

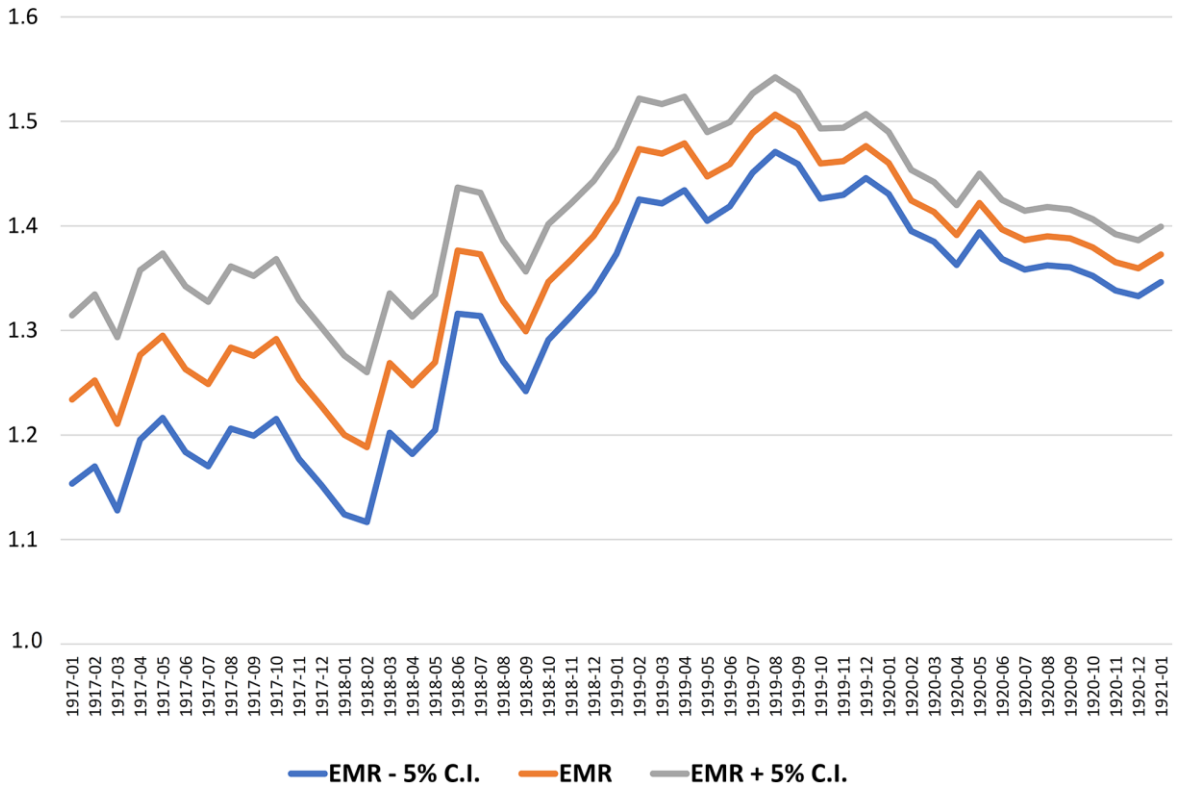

Figure 2. The EMR by month of birth with 5% confidence intervals (12 months moving average, 1917-1920).

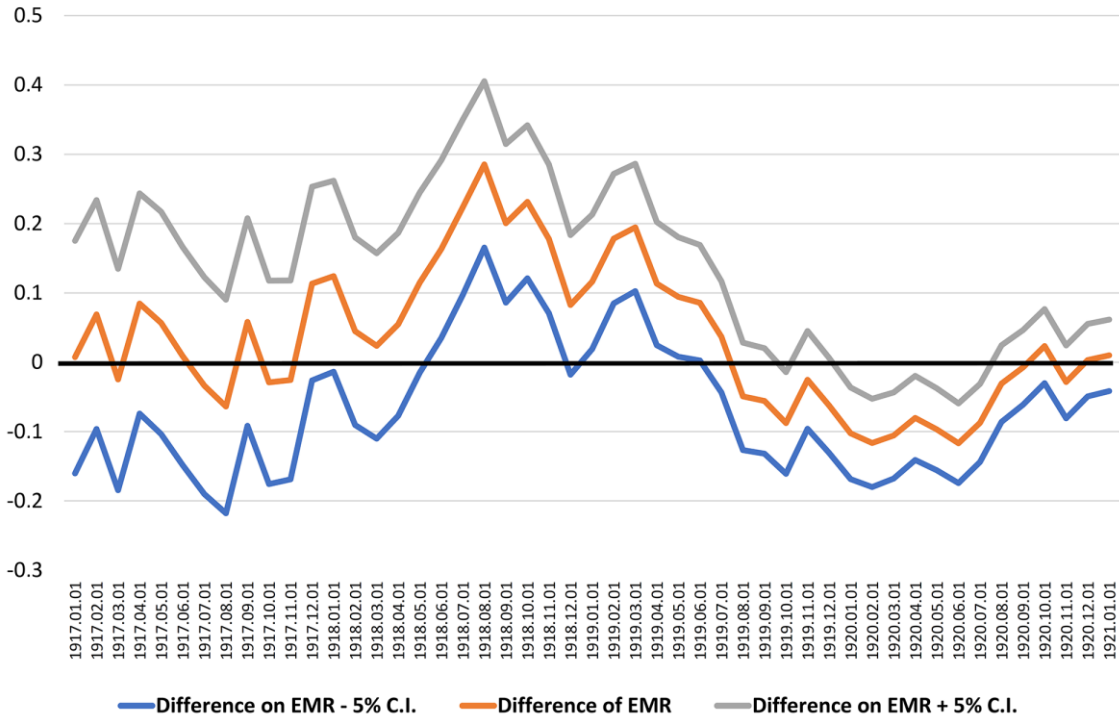

Figure 3. Identifying the best cut point as the larger difference of the EMR calculated between two adjacent periods of 12 months, from 1917-1920.

pandemic, compared with centenarians born later. The difference is statistically significant at a level of 5%. Such a relative survival advantage among the "older" centenarians is unexpected and intriguing enough to be worthy of further investigations.

Two main events, during the second half of 1918, may be associated to the EMR difference during the 2020 pandemic as reported in this article: the end of WWI on November 11, 1918 and the outbreak of the Spanish flu due to H1N1 influenza virus, which was attested in Belgium since July 1918.

The impact of the end of WWI

WWI (1914-1918) led to a period of deprivation for the Belgian population suffering under German occupation. Especially in 1917 and 1918, the nutritional status and sanitary conditions of the population had further worsened until 1920 [10]. The impact of nutrient deficit and low–quality foods during wartime may have had devastating effects on both the short– and long–term health of the new–born. Protein–energy malnutrition may heavily affect the course of pregnancies, causing increased stillbirth rates [11]. Unfortunately, no data on the number of stillborn and only little data on the level of infant mortality during WWI are available in Belgium [12]. An increase in infant mortality was observed in 1917 and 1918, a similar trend also reported in the neighboring countries, e.g., France and the Netherlands [13]. Such an increase may have resulted in a stronger selection against the weakest babies, born in this period, considering that harsh privations persisted more than a year after the end of the war. Research on French children born during WWI revealed that, in general, parental socioeconomic conditions were a strong predictor of new-borns' longevity [14]. Children whose mothers had faced famine during pregnancy tended to have a shorter lifespan [15]. These long-term effects have been ascribed to a direct fetal distress or mediated by epigenetic mechanisms [16]. Nevertheless, it is important to mention the strong support by the U.S. Commission for Relief in Belgium that improved the food supply for babies, thereby ensuring them better early–life conditions despite the poor socio-economic context lasting until the end of 1919 [17]. Although some kind of selection early in the life of these generations can hardly be excluded, its impact on their survival into old ages is unknown, and there is no consensus on the existence of its favorable or detrimental significance [18]. If such a selection was real and supposed to be favorable for survival, babies born during WWI or immediately after would be expected to have different mortality risks along their whole lifespan and not exclusively at the extreme end of life. To test the selection hypothesis, we disaggregated the mortality risks of babies born before and after the end of WWI, considering their month and year of births for the periods 1991-2000 and 2001-2010. The corresponding curves presented in Figure 4 do not show any evidence of a significant variation of mortality for people born around the end of WWI. We cogently conclude that a selection in terms of WWI can hardly be responsible for the relative better survival of 'older' centenarians compared with 'younger' ones against 2020 COVID-19, despite both groups likely experiencing similarly threatening early–life conditions during the three years around the end of WWI.

The impact of "Spanish flu"

The second important event that may potentially explain our results is the outbreak of the Spanish flu that caused about 50,000 deaths by the fall of 1918, i.e., more than the estimated 44,000 victims of WWI [19]. As explained by Brulard in his dissertation [20], the Belgian press covering the timeline of the Spanish flu mentioned the latter for the first time on July 7, 1918. The overall death rate in the city of Brussels increased: from July 1 to July 20, 68 civilians of the age 10–40 years died, while the number of deaths for the same age group in June was 35. In the first half of August, the whole country was facing the virus but, very quickly, the pandemic subsided, only to re-emerge in October. This second wave was by far the most devastating as more than half of the victims attributed to the Spanish flu died between mid-October and mid-November. Thereafter, it gradually lessened in intensity, although many deaths linked to the Spanish flu were still recorded in December. A third wave resurfaced in January 1919, culminating in February. Although it was less deadly than the second wave, it testified to the persistent circulation of the virus, which did not disappear until the end of May 1919.

In our analysis, we detect a synchronic association between the timing of the Spanish flu pandemic and the switch in centenarians' EMR. To better outline this potential association, we group the number of observed and expected deaths of centenarians during the 2020 pandemic into three periods of birth, namely, before, during and after the Spanish flu pandemic (Table 1). A significant difference in the EMR of centenarians in 2020 is evident between the "young" centenarians born after August 1, 1918 and the "older" ones born before that date.

The near-perfect synchronism between the EMR gap during the 2020 pandemic for cohorts born before and after August 1, 1918 and the surge of the influenza pandemic naturally suggests a causal effect, which becomes plausible as we excluded the main alternative

explanation based on the impact of WWI ending, especially considering the persistence of poor living conditions after the conflict.

For the exposure to the Spanish flu pandemic, three groups of newborns could be distinguished. The first group included babies who were already born before the pandemic broke out, and who faced the virus in their early life. The youngest among them could have been protected by maternal antibodies transferred through breastmilk during the first months of their life. Nonetheless, as the duration of the pandemic exceeded that of maternal protection, the majority of them had to face the virus. In contrast, babies born after August 1, 1918 but before the pandemic completely disappeared by the end of May 1919, could have been protected by maternal immunity, with a variable risk of coming in contact with the virus. Babies born after May 1919 might have been exposed to the H1N1 virus but only in utero and, if they survived, were protected by maternal immunity in their first months of life, while those born after January 1920 were not exposed to the virus at all.

Only the first group faced the virus expressing a significant survival advantage later in life during the 2020 COVID-19 pandemic. Our speculative hypothesis is that most of them could have developed immune memory cells capable of recognizing epitopes antigenically related to the H1N1 virus potentially even a century later. This hypothesis is supported by some scientific evidence. For instance, individuals born before 1957 and exposed to the H1N1 influenza A virus were better protected from the 2009 pH1N1 [21]. Even though the influenza and SARS-CoV-2 viruses are different, some structural homology between them has been reported [22] and a subset of the T cell repertoire capable of cross-reacting with both influenza virus and SARS-CoV-2 virus epitopes has been identified [23]. More specifically, SARS-CoV-2 T-cell repertoires with specificity to both the coronavirus and the M1 immunodominant epitope of influenza virus are more frequent that expected, which may have relevant implications in the response to COVID-19 for those individuals previously exposed to some influenza strains. Indeed, this raises the possibility that cohorts exposed to the Spanish flu in 1918 were capable to mount an effective response also against COVID-19 in 2020. Efficient memory cells can persist for many decades, as demonstrated by the study of Yu et al. [24] wherein individuals exposed to the H1N1 pandemic in

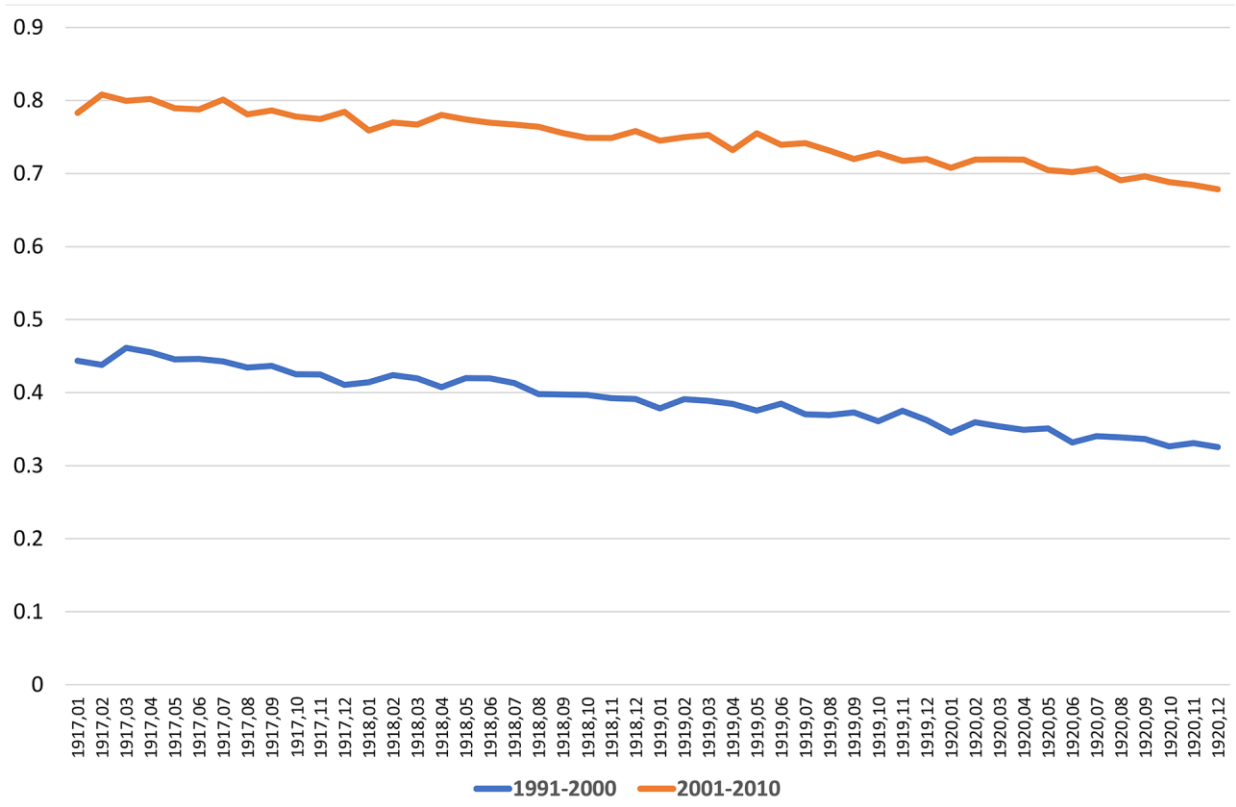

Figure 4. Probability of dying from 1991 until 2000 and from 2001 to 2010, calculated by year and month of births (STATBEL and data from Centre de Démographie, UCLouvain, Belgium).

Table 1. Number of expected and observed deaths of centenarians during the 2020 pandemic (March 10 – December 31) considering three periods, before, during and after the Spanish flu pandemic and estimated over-mortality.

Period of observation		At risk population March 10, 1918	Observed deaths	Expected deaths	Excess mortality ratio	5% confidence interval
January 1, 1917	July 31, 1918	501	180	144	125.0%	7.0%
August 1, 1918	May 31, 1919	370	138	96	143.8%	8.1%
June 1, 1919	December 31, 1920	2264	765	538	142.2%	3.4%

1918/1919 were still able to produce neutralizing antibodies a century later, confirming that a centenarian's immune system can successfully react to pathogens to which they were exposed early in life. However, this hypothesis is in contrast with that of Gagnon et al. [25] that exposure to the influenza pandemic early in life is a risk factor for dying during subsequent heterosubtypic pandemics. For this reason, a putative influenza / coronavirus cross-response should be viewed an interesting research hypothesis that will require further investigations aimed at better clarifying the underlying molecular mechanisms acting in the host.

Strengths, limitations, and future research

The main strength of the presented study is the direct link established between the two greatest pandemics of the past one hundred years. Our investigations are innovative in that we study the only persons exposed to both pandemics, i.e., today's centenarians. Fortunately, reliable and exhaustive data on centenarians and their survival is available in Belgium, and the sufficient number of people involved in our analysis allow a high level of statistical significance. Data concerning people born at the time of the Spanish flu and are still alive at the onset of the 2020 COVID-19 pandemic on March 10 are examined in great detail (according to month of birth), something that hardly occurs in such kind of research, taking into account the size of each birth cohort. Nevertheless, the study has several limitations. First, due to the small number of male centenarians, a separate analysis of males and females could not be performed. Another limitation is linked to the difficulty in distinguishing COVID-19-related deaths and those due to other causes. Further, only the overall mortality, including that due to COVID-19, is addressed as during the early stage of the pandemic, some cases are probably not attributed to COVID-19. Third, it cannot be ruled out that the susceptibility to COVID-19 among the oldest old is strongly influenced by the specific living conditions of this age group, as most centenarians were confined to nursing homes. Further research is ongoing to assess the impact of these collective living arrangements on mortality during the pandemic. Finally, we do not carry out direct blood testing in the oldest people experiencing COVID-19, therefore our hypothesis of protection provided by a cross-reactive immunity elicited during a previous H1N1 pandemic remains entirely speculative. Nevertheless, the possibility that those exposed to the Spanish flu have developed immune mechanisms capable of inducing a more effective anti-COVID-19 response than non-exposed people born after it is intriguing and deserves further investigation across different populations.

AUTHOR CONTRIBUTIONS

The first author conceives and designs the study having full access to all the data in the study. He is responsible for the integrity of the data and accuracy of the data analysis. MP drafts the manuscript. MP and GMP perform the analysis, and all authors including DC, critically review the manuscript for important intellectual content and give final approval for the version to be published.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

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REFERENCES

1. Yang L, Jin J, Luo W, Gan Y, Chen B, Li W. Risk factors for predicting mortality of COVID-19 patients: A systematic review and meta-analysis. PLoS One. 2020; 15:e0243124. https://doi.org/10.1371/journal.pone.0243124 PMID:33253244

2. Worldometers.info. Dover, Delaware, U.S.A. 2021. https://www.worldometers.info

3. Bustos Sierra N, Bossuyt N, Braeye T, Leroy M, Moyersoen I, Peeters I, Scohy A, Van der Heyden J, Van Oyen H, Renard F. All-cause mortality supports the COVID-19 mortality in Belgium and comparison with major fatal events of the last century. Arch Public Health. 2020; 78:117. https://doi.org/10.1186/s13690-020-00496-x PMID:33292536

4. Sciensano, Belgian Institute of Health. Brussels, Belgium. 2020. https://www.sciensano.be/en/covid-19-data

5. The Gerontology Wiki. https://gerontology.wikia.org/wiki/Julia_Van_Hool

6. Couderc AL, Correard F, Nouguerède E, Berbis J, Rey D, Daumas A, Villani P. Centenarians in nursing homes during the COVID-19 pandemic. Aging (Albany NY). 2021; 13:6247–57. https://doi.org/10.18632/aging.202743 PMID:33653968

7. Zerah L, Baudouin É, Pépin M, Mary M, Krypciak S, Bianco C, Roux S, Gross A, Toméo C, Lemarié N, Dureau A, Bastiani S, Ketz F, et al. Clinical Characteristics and Outcomes of 821 Older Patients With SARS-Cov-2 Infection Admitted to Acute Care Geriatric Wards. J Gerontol A Biol Sci Med Sci. 2021; 76:e4–12. https://doi.org/10.1093/gerona/glaa210 PMID:32845301

8. Marcon G, Tettamanti M, Capacci G, Fontanel G, Spanò M, Nobili A, Forloni G, Franceschi C. COVID-19 mortality in Lombardy: the vulnerability of the oldest old and the resilience of male centenarians. Aging (Albany NY). 2020; 12:15186–95. https://doi.org/10.18632/aging.103872 PMID:32788424

9. Statbel. La Belgique en chiffres. 2020. https://www.statbel.fgov.be/fr/open-data

10. Bernardo y. Garcia LA. Le Ventre des Belges : Une histoire alimentaire des temps d'occupation et de sortie de guerre (1914-1921 and 1939-1948). Archives générales du Royaume, ed. Bruxelles. 2017.

11. Davis G. Stillbirth registration and perceptions of infant death, 1900-60: the Scottish case in national context. Econ Hist Rev. 2009; 62:629–54. https://doi.org/10.1111/j.1468-0289.2009.00478.x PMID:20098665

12. Shapiro-Mendoza CK, Parks S, Lambert AE, Camperlengo L, Cottengim C, Olson C. The Epidemiology of Sudden Infant Death Syndrome and Sudden Unexpected Infant Deaths: Diagnostic Shift and other Temporal Changes. In: Duncan JR, Byard RW, editors. SIDS Sudden Infant and Early Childhood Death: The Past, the Present and the Future. Adelaide (AU): University of Adelaide Press. 2018. PMID:30035940

13. The Human Mortality Database. 2021. https://www.mortality.org

14. Todd N, Valleron AJ, Bougnères P. Prenatal loss of father during World War One is predictive of a reduced lifespan in adulthood. Proc Natl Acad Sci USA. 2017; 114:4201–06. https://doi.org/10.1073/pnas.1617911114 PMID:28377521

15. Bock MJ, Jarvis GC, Corey EL, Stone EE, Gribble KE. Maternal age alters offspring lifespan, fitness, and lifespan extension under caloric restriction. Sci Rep. 2019; 9:3138. https://doi.org/10.1038/s41598-019-40011-z PMID:30816287

16. Tobi EW, Slagboom PE, van Dongen J, Kremer D, Stein AD, Putter H, Heijmans BT, Lumey LH. Prenatal famine and genetic variation are independently and additively associated with DNA methylation at regulatory loci within IGF2/H19. PLoS One. 2012; 7:e37933. https://doi.org/10.1371/journal.pone.0037933 PMID:22666415

17. Buekens P, Debuisson M. Guerres mondiales et prévention maternelle et infantile : le cas de la Belgique. In: Child Health and Mortality in Europe. Chaire Quételet 1994. Louvain-la Neuve. 1996; 269–97.

18. Alter G. personal communication. 2021.

19. Lambert A. La population de la Belgique dans la guerre 1914-1918. 2014. https://adrass.net

20. Brulard B. La grippe espagnole en Belgique occupée (1918-1919): analyse épidémiologique et étude de l'imaginaire et de la perception de l'épidémie à travers les carnets de guerre. Université catholique de Louvain. 2018. http://hdl.handle.net/2078.1/thesis:16082

21. Lemaitre M, Leruez-Ville M, De Lamballerie XN, Salez N, Garrone P, Fluckiger AC, Klatzmann D, Carrat F.

Seasonal H1N1 2007 influenza virus infection is associated with elevated pre-exposure antibody titers to the 2009 pandemic influenza A (H1N1) virus. Clin Microbiol Infect. 2011; 17:732–37. https://doi.org/10.1111/j.1469-0691.2010.03352.x PMID:20731679

22. Takechi M, Nagasaki S, Nagasaki K. Relationships between Influenza viruses A and B and Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2): sequence homologies and implications for medicine treatment. OSF Preprints. 2020. https://doi.org/10.31219/osf.io/mjzyf

23. Sidhom JW, Baras AS. Analysis of SARS-CoV-2 specific T-cell receptors in ImmuneCode reveals cross-reactivity to immunodominant Influenza M1 epitope. bioRxiv. https://doi.org/10.1101/2020.06.20.160499

24. Yu X, Tsibane T, McGraw PA, House FS, Keefer CJ, Hicar MD, Tumpey TM, Pappas C, Perrone LA, Martinez O, Stevens J, Wilson IA, Aguilar PV, et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature. 2008; 455:532–36. https://doi.org/10.1038/nature07231 PMID:18716625

25. Gagnon A, Acosta JE, Madrenas J, Miller MS. Is antigenic sin always "original?" Re-examining the evidence regarding circulation of a human H1 influenza virus immediately prior to the 1918 Spanish flu. PLoS Pathog. 2015; 11:e1004615. https://doi.org/10.1371/journal.ppat.1004615 PMID:25742615

SUPPLEMENTARY MATERIALS

Supplementary Table

Supplementary Table 1. Population alive as of March 10, 2020 by year and month of birth, observed and expected number of deaths between March 10 and December 31.

Year	Month	Age as of March 10, 2020	Number of persons alive	Observed deaths	Observed mortality rate	Expected mortality rate	Expected deaths
1916	January	104 years 2 months	3	2	66,7%	31,4%	1
1916	February	104 years 1 month	10	4	40,0%	31,2%	3
1916	March	104 years	8	6	75,0%	31,1%	2
1916	April	103 years 11 months	16	7	43,8%	30,9%	5
1916	May	103 years 10 months	12	6	50,0%	30,8%	4
1916	June	103 years 9 months	10	3	30,0%	30,6%	3
1916	July	103 years 8 months	17	6	35,3%	30,4%	5
1916	August	103 years 7 months	13	3	23,1%	30,3%	4
1916	September	103 years 6 months	16	5	31,3%	30,1%	5
1916	October	103 years 5 months	9	2	22,2%	30,0%	3
1916	November	103 years 4 months	15	5	33,3%	29,8%	4
1916	December	103 years 3 months	14	5	35,7%	29,7%	4
1917	January	103 years 2 months	12	4	33,3%	29,5%	4
1917	February	103 years 1 month	16	10	62,5%	29,3%	5
1917	March	103 years	24	7	29,2%	29,2%	7
1917	April	102 years 11 months	10	4	40,0%	29,0%	3
1917	May	102 years 10 months	21	10	47,6%	28,9%	6
1917	June	102 years 9 months	14	5	35,7%	28,7%	4
1917	July	102 years 8 months	7	3	42,9%	28,6%	2
1917	August	102 years 7 months	17	2	11,8%	28,4%	5
1917	September	102 years 6 months	16	8	50,0%	28,3%	5
1917	October	102 years 5 months	18	6	33,3%	28,1%	5
1917	November	102 years 4 months	18	4	22,2%	28,0%	5
1917	December	102 years 3 months	20	6	30,0%	27,8%	6
1918	January	102 years 2 months	13	6	46,2%	27,7%	4
1918	February	102 years 1 month	24	12	50,0%	27,5%	7
1918	March	102 years	23	7	30,4%	27,4%	6
1918	April	101 years 11 months	20	5	25,0%	27,2%	5
1918	May	101 years 10 months	30	11	36,7%	27,1%	8
1918	June	101 years 9 months	17	4	23,5%	26,9%	5
1918	July	101 years 8 months	38	12	31,6%	26,8%	10
1918	August	101 years 7 months	40	15	37,5%	26,6%	11
1918	September	101 years 6 months	31	11	35,5%	26,5%	8
1918	October	101 years 5 months	23	9	39,1%	26,3%	6
1918	November	101 years 4 months	37	19	51,4%	26,2%	10
1918	December	101 years 3 months	39	12	30,8%	26,0%	10
1919	January	101 years 2 months	40	11	27,5%	25,9%	10
1919	February	101 years 1 month	40	14	35,0%	25,8%	10
1919	March	101 years	37	16	43,2%	25,6%	9
1919	April	100 years 11 months	38	13	34,2%	25,5%	10
1919	May	100 years 10 months	45	18	40,0%	25,3%	11
1919	June	100 years 9 months	47	18	38,3%	25,2%	12
1919	July	100 years 8 months	68	28	41,2%	25,0%	17
1919	August	100 years 7 months	58	20	34,5%	24,9%	14
1919	September	100 years 6 months	98	36	36,7%	24,8%	24
1919	October	100 years 5 months	102	32	31,4%	24,6%	25
1919	November	100 years 4 months	107	45	42,1%	24,5%	26

1919	December	100 years 3 months	118	45	38,1%	24,3%	29
1920	January	100 years 2 months	143	51	35,7%	24,2%	35
1920	February	100 years 1 month	112	36	32,1%	24,1%	27
1920	March	100 years	126	38	30,2%	23,9%	30
1920	April	99 years 11 months	134	46	34,3%	23,8%	32
1920	May	99 years 10 months	154	59	38,3%	23,6%	36
1920	June	99 years 9 months	161	51	31,7%	23,5%	38
1920	July	99 years 8 months	145	40	27,6%	23,4%	34
1920	August	99 years 7 months	153	46	30,1%	23,2%	36
1920	September	99 years 6 months	112	30	26,8%	23,1%	26
1920	October	99 years 5 months	151	57	37,7%	23,0%	35
1920	November	99 years 4 months	123	38	30,9%	22,8%	28
1920	December	99 years 3 months	152	49	32,2%	22,7%	34
1921	January	99 years 2 months	168	57	33,9%	22,5%	38
1921	February	99 years 1 month	168	50	29,8%	22,4%	38
1921	March	99 years	199	54	27,1%	22,2%	44
1921	April	98 years 11 months	197	56	28,4%	22,1%	44
1921	May	98 years 10 months	194	65	33,5%	22,0%	43
1921	June	98 years 9 months	191	62	32,5%	21,8%	42
1921	July	98 years 8 months	222	69	31,1%	21,7%	48
1921	August	98 years 7 months	239	74	31,0%	21,5%	51
1921	September	98 years 6 months	230	67	29,1%	21,4%	49
1921	October	98 years 5 months	232	67	28,9%	21,2%	49
1921	November	98 years 4 months	234	71	30,3%	21,1%	49
1921	December	98 years 3 months	280	81	28,9%	21,0%	59

Research Paper

Serum amylase elevation is associated with adverse clinical outcomes in patients with coronavirus disease 2019

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ABSTRACT

Objective: Hyperamylasemia was found in a group of patients with COVID-19 during hospitalization. However, the evolution and the clinical significance of hyperamylasemia in COVID-19, is not well characterized.
Design: In this retrospective cohort study, the epidemiological, demographic, laboratory, treatment and outcome information of 1,515 COVID-19 patients with available longitudinal amylase records collected from electronic medical system were analyzed to assess the prevalence and clinical significance of hyperamylasemia in this infection. Associated variables with hyperamylasemia in COVID-19 were also analyzed.
Results: Of 1,515 patients, 196 (12.9%) developed hyperamylasemia, among whom 19 (1.3%) greater than 3 times upper limit of normal (ULN) and no clinical acute pancreatitis was seen. Multivariable ordered logistic regression implied older age, male, chronic kidney disease, several medications (immunoglobin, systemic corticosteroids, and antifungals), increased creatinine might be associated with hyperamylasemia during hospitalization. Restricted cubic spline analysis indicated hyperamylasemia had a J-shaped association with all-cause mortality and the estimated hazard ratio per standard deviation was 2.85 (2.03-4.00) above ULN. Based on the multivariable mixed-effect cox or logistic regression model taking hospital sites as random effects, elevated serum amylase during hospitalization was identified as an independent risk factor associated with in-hospital death and intensive complications, including sepsis, cardiac injury, acute respiratory distress syndrome, and acute kidney injury.
Conclusions: Elevated serum amylase was independently associated with adverse clinical outcomes in COVID-19 patients. Since early intervention might change the outcome, serum amylase should be monitored dynamically during hospitalization.

INTRODUCTION

Owing to the emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), an outbreak of coronavirus disease 2019 (COVID-19) has violently spread almost all over the world. Carrying significant morbidity and mortality worldwide and posing an enormous threat to human beings, COVID-19 has developed into a global pandemic [1].

The COVID-19 pneumonia was primarily featured by fever, cough, fatigue, the cause of critical and even lethal lower respiratory tract infection, as well as extrapulmonary manifestations [2, 3]. An endeavor has been made by researchers to reveal the epidemiological, virological, and clinical characteristics of this pandemic [4–6]. However, most of the studies lay stress on illustrating respiratory symptoms, common complications, and significant risk factors of severe or deceased cases [7–9], while some non-classical but not insignificant morbidities or acute organ injury have been overlooked. For instance, our previous study indicated that a mild elevation of liver chemistries is most commonly found in patients with COVID-19 [10]. In addition, a portion of COVID-19 patients with serum amylase level elevation were observed in our clinical practice.

Previous studies attributed amylase abnormality to potential pancreatic damage caused by COVID-19 infection [11], which overemphasize the pancreatic source of amylase and overlook other possibility leading to hyperamylasemia. Studies of decades discovered that serum amylase levels depend on a balance between secretion and clearance [12]. Although recent research had identified its novel roles acting as promising diagnostic, therapeutic and prognostic biomarker applied to infection, cancer, and wound healing [13–15], none of previous studies with sufficient patients had evaluated the robust role of serum amylase in the COVID-19 progression. In addition, attribution elevated amylase in patients with COVID-19 to pancreatic injury is still a highly controversial issue [16, 17]. To address this concern, we designed and conducted this retrospective study to reveal the temporal and distributional patterns of serum hyperamylasemia in COVID-19 patients with a focus on its clinical significance and determinants.

MATERIALS AND METHODS

Study design and ethics

We conducted this retrospective study to investigate the clinical characteristics and outcomes of inpatients with COVID-19 who admitted to Tongji Hospital, a tertiary hospital designed by Chinese government for hospitalization of COVID-19 patients. All consecutive patients with a diagnosis of COVID-19 hospitalized in 3 different sites (Main District, Sino-French New City Branch, and Optical Valley Branch) of Tongji Hospital were included in this study. The study protocol was reviewed and approved by the Institutional Review Board of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology (Grant No. TJ-IRB-20200207).

Patient selection

COVID-19 was diagnosed according to 'Clinical management of severe acute respiratory infection when novel coronavirus (nCoV) infection is suspected: interim guidance' published by World Health Organization. Virological diagnosis was established by a positive result of transcription-polymerase chain reaction (RT-PCR) assay for SARS-CoV-2 nucleic acid from the nasal and pharyngeal swab specimens. We used the following inclusion and exclusion criteria to select patients. All consecutive virological-diagnosed COVID-19 patients who were subject to serious illness sufficient to admission in any branch of Tongji hospital between 18 January 2020 and 18 March 2020 were included in this study. Patients with any positive of the exclusion criteria as following were excluded: under 18 yr-old; absent of or with a duplicate medical record; a lack of core data (results of routine blood counts, blood tests of amylase, or chest CT imaging); with pregnancy, organ transplant history, AIDS, malignancy, acute fatal organ injury (e.g., acute myocardial infarction, acute coronary syndrome, acute pulmonary embolism, or acute stroke) or chronic organ failure (e.g., decompensated cirrhosis, decompensated chronic renal insufficiency, or severe congestive heart failure); with intraductal papillary mucinous neoplasms, or chronic pancreatitis. The detailed inclusion and exclusion criteria were summarized in Figure 1.

Data collection and patient follow-up

All data were extracted from the Tongji Cloud Hospital Information System (an electronic database of medical records). Three authors independently collected and double-checked the clinical demographic information, pre-existing morbidities, symptoms and vital signs, laboratory examinations, radiological findings at admission, treatment and clinical outcomes during hospitalization of the patients with standardized forms. Before data extraction, personal identification information (e.g., name, medical ID) were removed and anonymized with an electronic code out of patient privacy protection. The pre-existing comorbidities were recorded by the physicians as part of routine clinical care based on patient self-report and medical history, and categorized according to ICD-10 coding.

All patients were followed up from the day at admission until they reached definite primary endpoint (discharge from or death in the hospital) and no patient was lost to follow up. The median follow-up time was 21 [IQR, 12-33] days. On 23 April 2020, the final date of follow up, we finished data extraction and started analysis and all patients reach primary endpoint.

Outcomes and definitions

The observation period of the time-vary variables was the duration between hospital admission and composite endpoint. The patients who presented with a peak value of serum amylases above ULN during illness were categorized into ESA (elevated serum amylase) group, while their counterparts with normal peak values into non-ESA group. The primary outcome was in-hospital death and secondary outcomes were the incidences of common critical complications during hospitalization, such as SARS-CoV-2 related acute respiratory distress syndrome (ARDS), acute kidney injury (AKI), sepsis, acute cardiac injury, and disseminated intravascular coagulation (DIC).

The diagnosis of hyperamylasemia or elevated serum amylase is established with an elevated serum amylase beyond ULN. The ULN of serum amylase (115 U/L) was determined by determined at the clinical laboratory of Tongji Hospital. Diagnosis of acute pancreatitis was based on Chinese guidelines for the management of acute pancreatitis (Shenyang, 2019) [18]. In detail, the diagnosis of acute pancreatitis requires at least two of the following three features: (1) abdominal pain consistent with acute pancreatitis (acute onset of a persistent, severe, epigastric pain often radiating to the back); (2) serum lipase activity (or amylase activity) at least three times greater than the upper limit of normal; and (3) characteristic findings of acute pancreatitis on contrast-enhanced computed tomography (CECT) and less commonly magnetic resonance imaging (MRI) or abdominal ultrasonography.

The definition of ARDS and sepsis was according to the interim guidance of WHO. We defined the acute kidney injury according to an elevation in serum creatinine (SCr) by ≥0.3 mg/dl (≥26.5 μmol/l) within 48 hours or SCr to ≥1.5 times baseline within the prior 7 days.

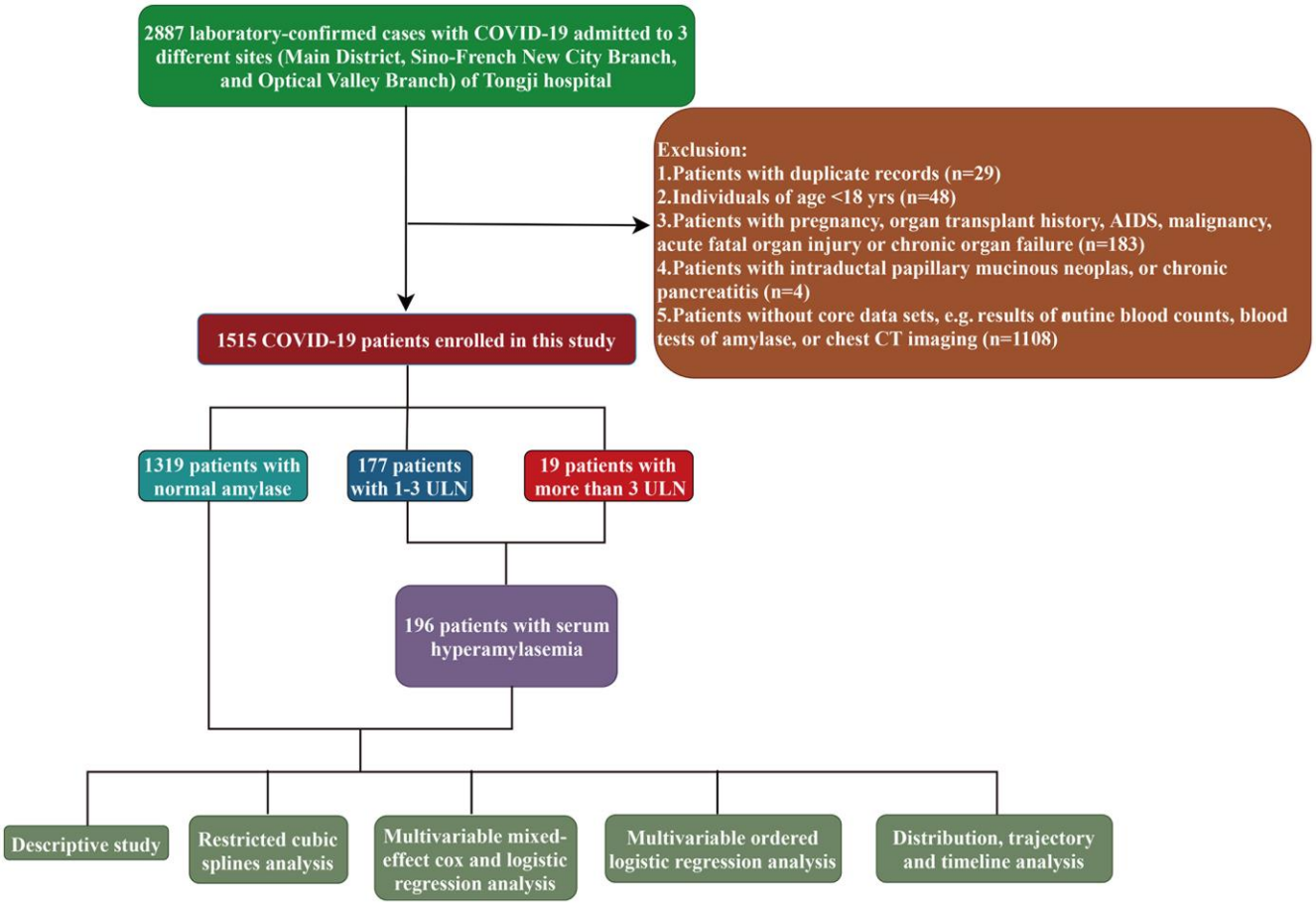

Figure 1. The flowchart showing enrollment of participants in this study.

Cardiac injury was defined by concentrations of any cardiac biomarkers (e.g., cardiac troponin I (cTNI), cardiac troponin T (cTNT), or high sensitivity cardiac troponin I (hs-cTNI)) higher than the upper limit of the normal range [9]. The diagnosis of DIC was established on the basis of the criteria illustrated in the International Society on Thrombosis and Hemostasis [19]. Definition of hyperlipidemia was according to 2019 ESC/EAS Guidelines for the management of dyslipidaemias [20].

Public RNA-Seq data

RNA-Seq dataset originated from the Genotype Tissue Expression Project (GTEx), corresponding to 8,555 samples from 31 normal human tissues, was downloaded from UCSC Xena [21]. Using Toil, UCSC's pipeline architecture, the expression of total genes was recomputed to create a consistent meta-analysis of the dataset free of computational batch effects [22]. Transcripts Per Kilobase of exon model per Million mapped reads (TPM) values of *ACE2* and *TMPRSS2* was extracted, and log2(TPM+0.001) transformed.

Statistical analysis

Continuous variables were provided as median (interquartile range [IQR]) and compared with independent group t tests or Mann-Whitney test depending on whether the data were normal distribution. Categorical data were presented as absolute count (percentage) and were compared with the Chi-square test or Fisher exact test. Distribution of peak amylase by the mortality of COVID-19 was evaluated by kernel density estimation and the dynamic changes of peak amylase by the mortality of COVID-19 were predicting using Generalized Additive Models (GAM) [23]. Adjusted hazard ratios (HR) or odds ratios(OR) and 95% confidence interval (95% CI) were calculated based on multivariable mixed-effect Cox proportional hazard [24] or mixed-effect logistic regression model [25] taking sites (hospital branches) as random effects by adjusting for age > 65-yr-old, sex, body mass index, gastrointestinal symptoms (including anorexia, nausea or vomiting, diarrhea, abdominal pain), comorbidities (hypertension, diabetes, coronary artery disease, cerebrovascular disease, chronic kidney disease, chronic liver disease, and chronic pulmonary disease), and severity of COVID-19 in the crude cohorts. Kaplan-Meier survival analysis was used to illustrate the cumulative rate of in-hospital mortality [26]. Ordered logistic regression analysis with the negative log-log link acting as the increasing function was also conducted to reveal the correlation of baseline clinical characteristics and medications happened before peaking of amylase in the longitudinal cohort, where serum amylase was trichotomized. Restricted cubic spline analysis with three knots at the 5th, 50th, and 95th centiles, which could make model flexible, was used to evaluate whether the correlation between serum amylase and COVID-19 mortality was linear with the reference value (OR=1) at 115 U/L for serum amylase concentration [27]. Interaction contrast ratios (ICR) were calculated to assess the additive interaction between serum amylase and common clinical characteristics in the cox regression [28]. No variables with missing data were used for aforementioned regression analysis so there is no need to made imputation for the missing data. All statistical tests were two-sided and statistical significance was taken as $p<0.05$. All statistical analyses were conducted using R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

Ethics approval

The study protocol was reviewed and approved by the Institutional Review Board of Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology (Grant No. TJ-IRB-20200207).

Data availability statement

Data are available on reasonable request.

RESULTS

Descriptions of cohort

Altogether 1515 laboratory-confirmed COVID-19 patients were analyzed in this study, among whom 196 presented with serum amylase level elevation, 19 (19/196, 9.7%) greater than 3-fold of ULN (>3ULN; >345 U/L) during hospitalization. None of patients with serum amylase higher than 3-fold of ULN developed abdominal pain during hospitalization. According to Revised Atlanta Classification [16, 29], the clinical manifestations and limited available abdominal imaging examinations in our study indicated that no acute pancreatitis diagnosis was established. The clinical characteristics and outcomes of those with serum amylase >345U/L were presented in Supplementary Table 1.

Compared with individuals in non-ESA group (Table 1), those with elevated serum amylases levels were older (median [IQR], 66 [56, 73] vs 60 [49, 68] years) and with a larger proportion of males (66.8% vs 45.9%). Cough, fatigue and dyspnea were significantly more prevalent in ESA group than and in non-ESA group, while no significant difference was noted between the two groups for gastrointestinal symptoms (any symptoms of anorexia, nausea or vomiting, diarrhea, and abdominal pain) (41.3% vs 41.8%). Their baseline characteristics,

Table 1. Basic characteristics of COVID-19 patients with or without serum amylase abnormality.

Characteristic	Serum amylase level			
	Overall	Elevated	Normal	*P* value
	N=1515	N=196	N=1319	
Age- yr	61 [49, 69]	66 [56, 73]	60 [49, 68]	<0.001
Age≥ 65	602 (39.7)	114 (58.2)	488 (37.0)	<0.001
Male	737 (48.6)	131 (66.8)	606 (45.9)	<0.001
BMI	23.9 [22.1, 25.7]	23.7[21.8, 25.5]	23.9 [22.2, 25.7]	0.12
Time from illness onset to hospital admission, days	14 [9, 24]	11.50 [7, 18]	15 [9, 25.6]	<0.001
Severe pneumonia (NHC)*	652 (43.0)	112 (57.1)	540 (40.9)	<0.001
Signs and symptoms				
Fever	1132 (74.7)	155 (79.1)	977 (74.1)	0.16
Cough	1086 (71.7)	157 (80.1)	929 (70.4)	0.01
Fatigue	449 (29.6)	72 (36.7)	377 (28.6)	0.03
Chest pain	103 (6.8)	10 (5.1)	93 (7.1)	0.39
Gastrointestinal symptoms**	633 (41.8)	81 (41.3)	552 (41.8)	0.95
Dyspnea	524 (34.6)	84 (42.9)	440 (33.4)	0.01
Myalgia	247 (16.3)	33 (16.8)	214 (16.2)	0.91
Ascites	3 (0.2)	1 (0.5)	2 (0.2)	0.847
Vital signs				
Respiratory rate, breaths per minute	20 [20, 22]	20 [20, 24]	20 [20, 22]	0.01
Pulse, beat per minute	85 [78, 97]	86 [78, 99]	85 [78, 97]	0.17
Mean arterial pressure, mmHg	97 [89, 105]	97[90, 104]	97 [89, 105]	0.47
Percutaneous oxygen saturation	97 [95, 98]	96 [92, 98]	97 [95, 98]	<0.001
Pre-existing comorbidity				
Chronic liver disease	121 (8.0)	21 (10.7)	100 (7.6)	0.17
Cardio-cerebrovascular metabolic diseases$	542 (35.8)	93 (47.4)	449 (34.0)	<0.001
Chronic pulmonary disease#	91 (6.0)	15 (7.7)	76 (5.8)	0.38
Chronic kidney disease	57 (3.8)	18 (9.2)	39 (3.0)	<0.001

1. Data were provided as number (percentage), median (interquartile range).

2. Serum Amylase Level, in a healthy individual, a normal blood amylase level ranges from 0-115 units per liter (U/L) in our hospital. The normal group included patients with normal serum amylase level, while the elevated group with serum amylase level >115U/L.

3. Severe pneumonia (NHC)*, the illness severity was classified according to Guidance for Corona Virus Disease 2019 (6/7th edition) released by the National Health Commission of China; Gastrointestinal symptoms**, including anorexia, nausea or vomiting, diarrhea, abdominal pain; Chronic pulmonary diseases# contain chronic obstructive pulmonary disease, asthma, bronchiectasis and pulmonary fibrosis; Cardio-cerebrovascular metabolic diseases$ includes hypertension, cardiovascular disease, cerebrovascular disease, diabetes.

4. Abbreviations: COVID-19, coronavirus disease 2019; SMD, standard mean difference; BMI, body mass index, which was calculated as weight in kilograms divided by height in meters squared.

5. Comorbidities diagnoses are established by medical history and classified according to ICD-10 coding. These include, but are not limited to, those shown in the table.

pre-existing morbidities, clinical symptoms and vital signs at admission were provided in Table 1.

Patients in ESA group presented with significantly more frequent and prominent abnormalities in laboratory findings than in non-ESA group (Table 2), including complete blood count, coagulation function, liver and kidney function, myocardial injury marker, and inflammatory parameters. Moreover, patients with hyperamylasemia were more prone to presented with bilateral involvement in radiological imaging than those with normal serum amylases (93.9% vs 83.6%). In

Table 2. Laboratory findings and radiological features of COVID-19 patients with or without serum amylase abnormality.

Laboratory findings	Normal range	Serum amylase level			*P*
		Overall	Elevated	Normal	
		N=1515	N=196	N=1319	
Blood routine					
Leukocyte count, $\times10^9$ per L	3·5-9·5	5.86 [4.52, 7.70]	6.50 [4.74, 8.87]	5.77 [4.50, 7.47]	<0.001
>9·5		188/1515 (12.4)	45/196 (23.0)	143/1319 (10.8)	<0.001
Neutrophil count, $\times10^9$ per L	1·8-6·3	3.79 [2.66, 5.46]	4.93 [3.16, 7.35]	3.64 [2.61, 5.28]	<0.001
>6·3		274/1515 (18.1)	66/196 (33.7)	208/1319 (15.8)	<0.001
Lymphocyte count, $\times10^9$ per L	1·1-3·2	127.00 [115.00, 138.00]	129.00 [116.00, 139.25]	127.00 [115.00, 138.00]	0.61
<1·1		340/1515 (22.4)	46/196 (23.5)	294/1319 (22.3)	0.78
Platelet count, $\times10^9$ per L	125-350	1.19 [0.78, 1.64]	0.84 [0.60, 1.23]	1.24 [0.85, 1.68]	<0.001
<125		689/1515 (45.5)	132/196 (67.3)	557/1319 (42.2)	<0.001
Coagulation function					
Prothrombin time, s	11·5-14·5	13.80 [13.20, 14.40]	14.00 [13.40, 15.00]	13.70 [13.12, 14.30]	<0.001
>14·5		321/1505 (21.3)	72/195 (36.9)	249/1310 (19.0)	<0.001
D-dimer, ug/ml FEU	<0·5	0.65 [0.29, 1.63]	1.38 [0.58, 3.36]	0.58 [0.26, 1.43]	<0.001
>0·5		851/1494 (57.0)	152/193 (78.8)	699/1301 (53.7)	<0.001
Liver function					
Aspartate aminotransferase, U/L	≤40	25.00 [18.00, 37.00]	32.00 [22.00, 47.25]	24.00 [18.00, 35.00]	<0.001
>40		317/1515 (20.9)	70/196 (35.7)	247/1319 (18.7)	<0.001
Alanine aminotransferase, U/L	≤41	22.00 [14.00, 38.00]	25.00 [16.75, 38.00]	22.00 [14.00, 38.00]	0.02
>41		342/1515 (22.6)	45/196 (23.0)	297/1319 (22.5)	0.96
Total bilirubin, μmol/L	≤21	8.70 [6.40, 12.10]	9.30 [7.00, 13.90]	8.60 [6.40, 11.95]	0.01
>21		73/1515 (4.8)	22/196 (11.2)	51/1319 (3.9)	<0.001
Direct bilirubin, μmol/L	≤8·0	3.70 [2.70, 5.20]	4.40 [3.10, 6.40]	3.60 [2.70, 5.05]	<0.001
>8·0		128/1515 (8.4)	34/196 (17.3)	94/1319 (7.1)	<0.001
Hyperlipidemia*		100 (6.6)	10 (5.1)	90 (6.8)	0.452
Renal function					
Creatinine, μmol/L	45-84	69.00 [57.00, 83.00]	80.00 [64.75, 100.25]	67.00 [56.00, 81.00]	<0.001
>84		354/1515 (23.4)	83/196 (42.3)	271/1319 (20.5)	<0.001
Blood urea nitrogen, mmol/L	1·7-8·3	4.50 [3.50, 5.85]	5.50 [4.00, 7.95]	4.40 [3.50, 5.60]	<0.001
>8·3		140/1515 (9.2)	46/196 (23.5)	94/1319 (7.1)	<0.001
eGFR, ml/min/1·73m2	>90	92.30 [73.55, 103.10]	79.00 [46.40, 101.10]	92.90 [75.57, 103.40]	<0.001
<90		691/1507 (45.9)	115/195 (59.0)	576/1312 (43.9)	<0.001
Cardiac markers					
Creatinine kinase, U/L	≤190	61.00 [41.00, 99.00]	74.00 [45.00, 146.00]	60.00 [40.00, 96.00]	<0.001
>190		119/1230 (9.7)	36/169 (21.3)	83/1061 (7.8)	<0.001
High-sensitivity cardiac troponin I	≤15·6	3.80 [1.00, 9.60]	7.60 [3.05, 25.80]	3.30 [1.00, 8.07]	<0.001
>15·6		211/1249 (16.9)	57/171 (33.3)	154/1078 (14.3)	<0.001
N-terminal pro-brain natriuretic peptide	<486	106.00 [35.00, 325.25]	259.00 [76.00, 1209.00]	91.00 [31.00, 282.00]	<0.001
>486		233/1252 (18.6)	63/171 (36.8)	170/1081 (15.7)	<0.001
Inflammation makers					
High sensitivity C-reactive protein, mg/L	<10	12.30 [2.00, 57.80]	48.50 [12.45, 105.85]	10.10 [1.70, 47.95]	<0.001
≥10		808/1511 (53.5)	149/196 (76.0)	659/1315 (50.1)	<0.001
Interleukin-6, pg/ml	<7.0	5.31 [2.08, 21.18]	15.02 [4.99, 55.25]	4.67 [1.91, 18.50]	<0.001
≥7.0		633/1402 (45.1)	126/182 (69.2)	507/1220 (41.6)	<0.001
Chest CT on admission					
Bilateral lesion		1287 (85.0)	184 (93.9)	1103 (83.6)	<0.001

1. Data were provided as number (percentage), median (Interquartile range).
2. The normal range of laboratory parameters were based on Tongji hospital and might vary in different hospitals.

addition, four of 19 patients with serum amylase greater than 3 times of ULN underwent abdominal CT imaging and no evident abnormality were found by experienced radiologists.

Individuals with serum amylase level elevation during hospitalization require more intensive integrated in-hospital treatment to manage COVID-19 (Table 3). Overall, individuals in EAS group censored a more frequent requirement for antibiotics (90.3% vs 74.1%), antifungal drugs (11.7% vs 2.7%), systemic corticosteroids (75.0% vs 43.7%), renal replacement therapy (15.8% vs 1.7%), as well as administration of mechanical ventilation (40.3% vs 13.6%), either non-invasively or invasively.

Complications and in-hospital mortality in COVID-19 patients

As presented in Table 4, COVID-19 patients with elevated serum amylases usually developed complications of acute kidney injury, ARDS, acute heart failure, and cardiac injury, the incidence was significantly higher in those with normal serum amylases (34.2% vs 5.1%, 45.9% vs 16.7%, 43.9% vs 13.1%, and 56.1% vs 21.7%), respectively.

Overall 118 in-hospital death occurred in our follow-up period, among whom 46 (23.5%) presented with abnormalities in serum amylase versus 72 (5.5%) with normal range. Unfortunately, 16 (84.2) patients with serum amylase higher than 3-fold of ULN were admitted in intensive care unit and 78.9% (15/19) died of COVID-19 in hospital.

Associations between elevated serum amylase and mortality, as well as secondary outcomes

Restricted cubic splines analysis was performed to flexibly model and visualize the association between serum amylase and all-cause mortality in COVID-19 patients. Regarding strong J-shaped association between serum amylase and all-cause mortality, the plot illustrated a substantial reduction of the risk within the lower range of serum amylase, which reached the lowest risk around 115 U/L and increased thereafter (*P* for non-linearity <0.001, Figure 2A). Above 115 U/L, the hazard ratio per standard deviation higher serum amylase calculated by mixed-effect Cox model was 2.85 (95% CI, 2.03 to 4.00).

In addition, mixed-effect Cox model was also constructed to assess the correlation between serum amylase and all-cause mortality. As a result, hyper-amylasemia was highly associated to mortality in COVID-19 patients (Figure 2B, 1-3 times ULN: HR=2.29, 95% CI [1.49, 3.51], *P*<0.001; > 3ULN: HR=12.96, 95% CI [7.38, 22.76], *P*<0.001). After adjusting for age, gender, BMI, gastrointestinal symptoms, pre-existing comorbidities, and severity of COVID-19, hyperamylasemia remained an independent risk factor for mortality (1-3 times ULN: HR=1.63, 95% CI [1.04, 2.55], *P*=0.034; >3ULN: HR=8.90, 95% CI [4.96,15.97], *P*<0.001). In addition, based on multivariable mixed-effect logistic regression model, hyperamylasemia was identified to be independently associated with sepsis (Figure 2C, 1-3 times the ULN: OR=1.15, 95% CI [1.11,1.22], *P*<0.001; >3ULN: OR=1.87, 95% CI [1.64,2.15], *P*<0.001), DIC (1-3 times ULN: OR=1.13, 95% CI [1.10,1.17], *P*<0.001; > 3ULN: OR=1.65, 95% CI [1.50,1.81], *P*<0.001), cardiac injury(1-3 times ULN: OR=1.24, 95% CI [1.16,1.34], *P*<0.001; > 3ULN: OR=1.71, 95% CI [1.42,2.07], *P*<0.001), ARDS(1-3 times ULN: OR=1.21, 95% CI [1.14,1.28], *P*<0.001; > 3ULN: OR=1.62, 95% CI [1.38,1.92], *P*<0.001) and AKI(1-3 times ULN: OR=1.24, 95% CI [1.19,1.30], *P*<0.001; > 3ULN: OR=1.79, 95% CI [1.59,2.02], *P*<0.001).

Associations between clinical characteristics and medications with serum amylase level

With Cox regression model, ICR was calculate to assess the additive interaction between serum amylase and common clinical characteristics. The results indicated that there is no significance in additive interaction and no need to perform subgroup analysis to evaluate associations between clinical characteristics and serum amylase (Supplementary Figure 1). However, multivariable ordered logistic regression analysis revealed the effects of common clinical characteristics on peak amylase levels in COVID-19 patients from the longitudinal cohort, and the results indicated that older age, male, chronic kidney disease, several medications (immunoglobin, systemic corticosteroids, and antifungals) and increased creatinine might be independently associated with the elevated amylase levels during hospitalization in COVID-19 patients (Figure 3).

Dynamic profile of serum amylase level in COVID-19 patients

In order to ascertain distribution and trajectory of serum amylase in COVID-19 patients, multiple results from different days were recorded during hospitalization. By kernel density estimation, different serum amylase distributions were investigated between survivors and those who died of COVID-19. Amylase levels were lower and less disperse in survived cases, on contrast, the levels increased and grew more disperse in deceased patients (Figure 4A). GAM model was constructed to explicate dynamic trajectories of serum amylase in

Table 3. Treatments in hospital of COVID-19 patients with or without serum amylase abnormality.

	Serum amylase level			*P* value
	Overall	Elevated	Normal	
	N=1515	N=196	N=1319	
Treatments in hospital				
Antiviral therapy	1429 (94.3)	189 (96.4)	1240 (94.0)	0.23
Antibiotics	1154 (76.2)	177 (90.3)	977 (74.1)	<0.001
Antifungal drugs	58 (3.8)	23 (11.7)	35 (2.7)	<0.001
NSAIDs	203 (13.4)	46 (23.5)	157 (11.9)	<0.001
Systemic corticosteroids	723 (47.7)	147 (75.0)	576 (43.7)	<0.001
Intravenous immunoglobin	583 (38.5)	120 (61.2)	463 (35.1)	<0.001
Traditional Chinese Medicine treatment	1299 (85.7)	167 (85.2)	1132 (85.8)	0.90
Proton pump inhibitor	694 (45.8)	132 (67.3)	562 (42.6)	<0.001
somatostatin analog	3 (0.2)	1 (0.5)	2 (0.2)	0.847
Fasting or parenteral nutrition	251 (16.6)	62 (31.6)	189 (14.3)	<0.001
Administration of mechanical ventilation	258 (17.0)	79 (40.3)	179 (13.6)	<0.001
Non-invasive	127 (8.4)	27 (13.8)	100 (7.6)	0.01
Invasive	131 (8.6)	52 (26.5)	79 (6.0)	<0.001
Admission to intensive care unit	156 (10.3)	63 (32.1)	93 (7.1)	<0.001
Renal replacement therapy	54 (3.6)	31 (15.8)	23 (1.7)	<0.001
Extracorporeal membrane oxygenation	18 (1.2)	12 (6.1)	6 (0.5)	<0.001

Data were provided as number (percentage), median (interquartile range).

Table 4. Clinical outcomes of COVID-19 patients with or without serum amylase abnormality.

	Serum amylase level			*P* value
	Overall	Elevated	Normal	
	N=1515	N=196	N=1319	
Clinical outcomes				
In-hospital death	118 (7.8)	46 (23.5)	72 (5.5)	<0.001
Duration from illness onset to death, days	25 [19, 36]	29 [21, 41]	25 [19, 34]	0.11
Hospital discharge	1397 (92.2)	150 (76.5)	1247 (94.5)	<0.001
Duration from illness onset to discharge, days	42 [32, 54]	45 [34, 56]	42 [32, 54]	0.06
Complications				
Acute kidney injury	134 (8.8)	67 (34.2)	67 (5.1)	<0.001
Acute respiratory distress syndrome	310 (20.5)	90 (45.9)	220 (16.7)	<0.001
Acute heart failure	217 (17.3)	75 (43.9)	142 (13.1)	<0.001
Cardiac injury	330 (26.4)	96 (56.1)	234 (21.7)	<0.001
Sepsis	174 (11.5)	62 (31.6)	112 (8.5)	<0.001
Disseminated intravascular coagulation	77 (5.2)	41 (21.2)	36 (2.8)	<0.001

Data were provided as number (percentage), median (interquartile range).

both survived and deceased cases during hospitalization (Figure 4B). The results indicated amylase level was higher in the deceased cases than in those who recover from COVID-19 infection. The fluctuation in survivors was mild and their amylase level predominantly maintained in the normal range. Nonetheless, the amylase level in deceased cases gradually increased, surpassed the ULN, and reached its peak within 18 to 22 days.

The timeline with death, discharge and different amylase levels marked (Figure 5), was performed to further assess the time-dependent development of hyperamylasemia events. Amylase level in the terminal time between survived and deceased patients were significantly different, which indicated that 11 (26.8%), 16 (39.0%), and 14 (34.1%) of 41 patients deceased with higher than 3 times ULN, 1-3 times ULN, and normal amylase level, separately, whereas, 0, 47 (53.4%), and 41 (46.6%) of 88 patients survived with higher than 3 times ULN, 1-3 times ULN, and normal level. This dynamic profile contributed to identify potential mechanisms of COVID-19-associated hyperamylasemia.

DISCUSSION

Detrimental complication, along with multi-organ dysfunction, which evidently associated with in-hospital mortality, is not uncommon in COVID-19 patients [10]. Our study illustrated trajectories of serum amylase levels in hospitalized COVID-19 patients and portrayed their clinical significance based on comprehensive analysis in a large retrospective cohort of 1515 participants. The deceased cases manifested with greater fluctuation in serum amylase level, in contrast, the variation in survivors was mild and primarily maintained in the normal range. Our study first reported the association between serum amylase level elevation and in-hospital mortality in a large cohort with comprehensive analysis after adjusting for potential cofounders. Moreover, our study discriminated that older age, male gender, chronic kidney disease, several medications (NSAIDs, immunoglobin, systemic corticosteroids, and antifungals) and increased creatinine might be independently associated with the elevated amylase levels during hospitalization in COVID-19 patients.

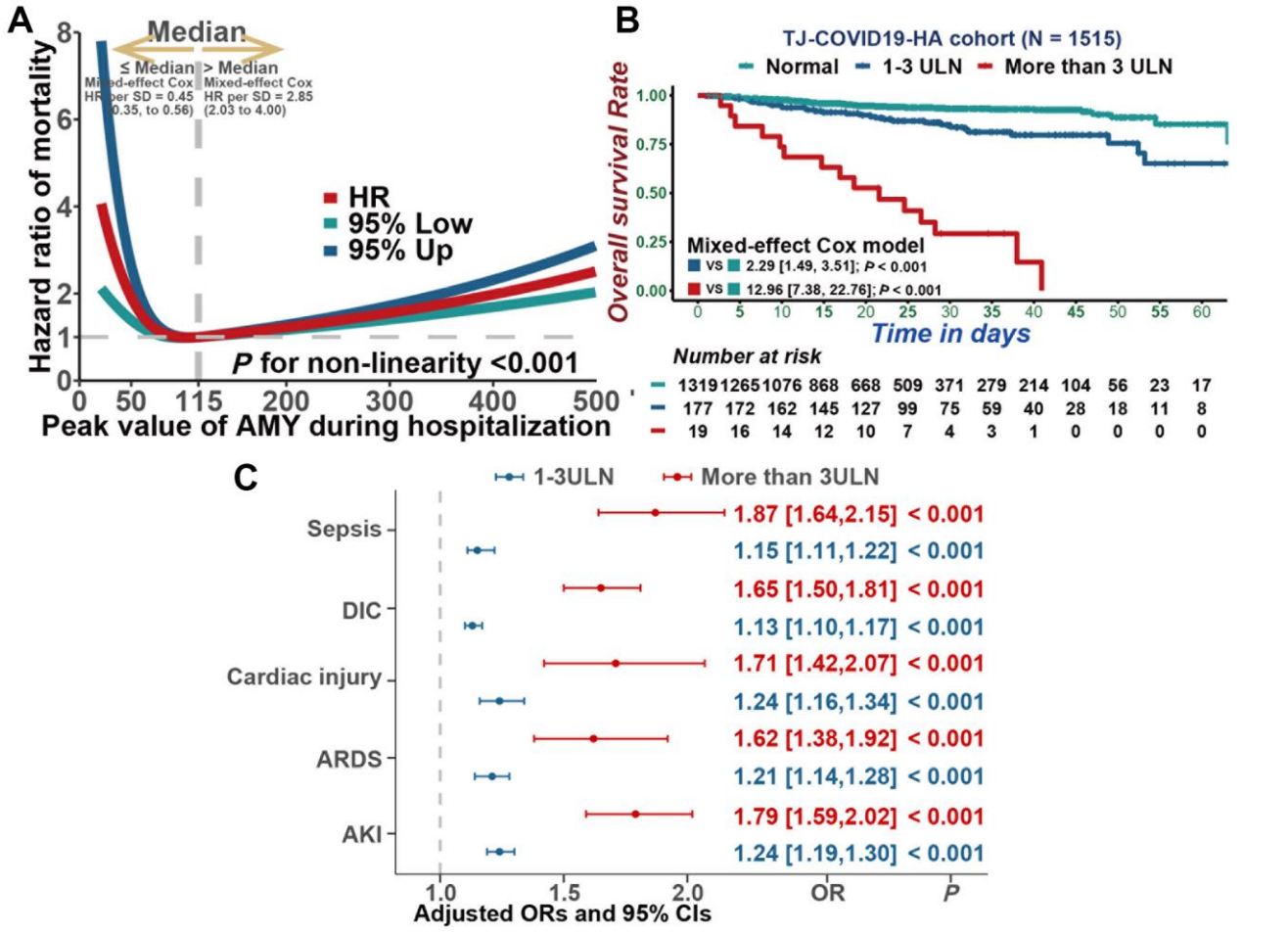

Figure 2. Associations between serum amylase and mortality, as well as secondary outcomes. (**A**) Restricted cubic splines analysis illustrated the association between serum amylase and all cause mortality. (**B**) Kaplan-Meier curves for cumulative probability of COVID-19 mortality during hospitalization in patients with different level of serum amylase. (**C**) Multivariable mixed-effect logistic regression model indicated that hyperamylasemia was independently associated with sepsis, DIC, Cardiac injury, ARDS and AKI.

A previous study reported 17% of COVID-19 patients presented with some form of pancreatic injury, based on any abnormality in serum amylase or lipase [11]. In the aforementioned study, of the all documented 52 COVID-19 patients, 13.5% (7/52) had elevated serum amylase, which share the similar incidence of serum hyperamylasemia with our data (12.9%). This retrospective study attributed hyperamylasemia to

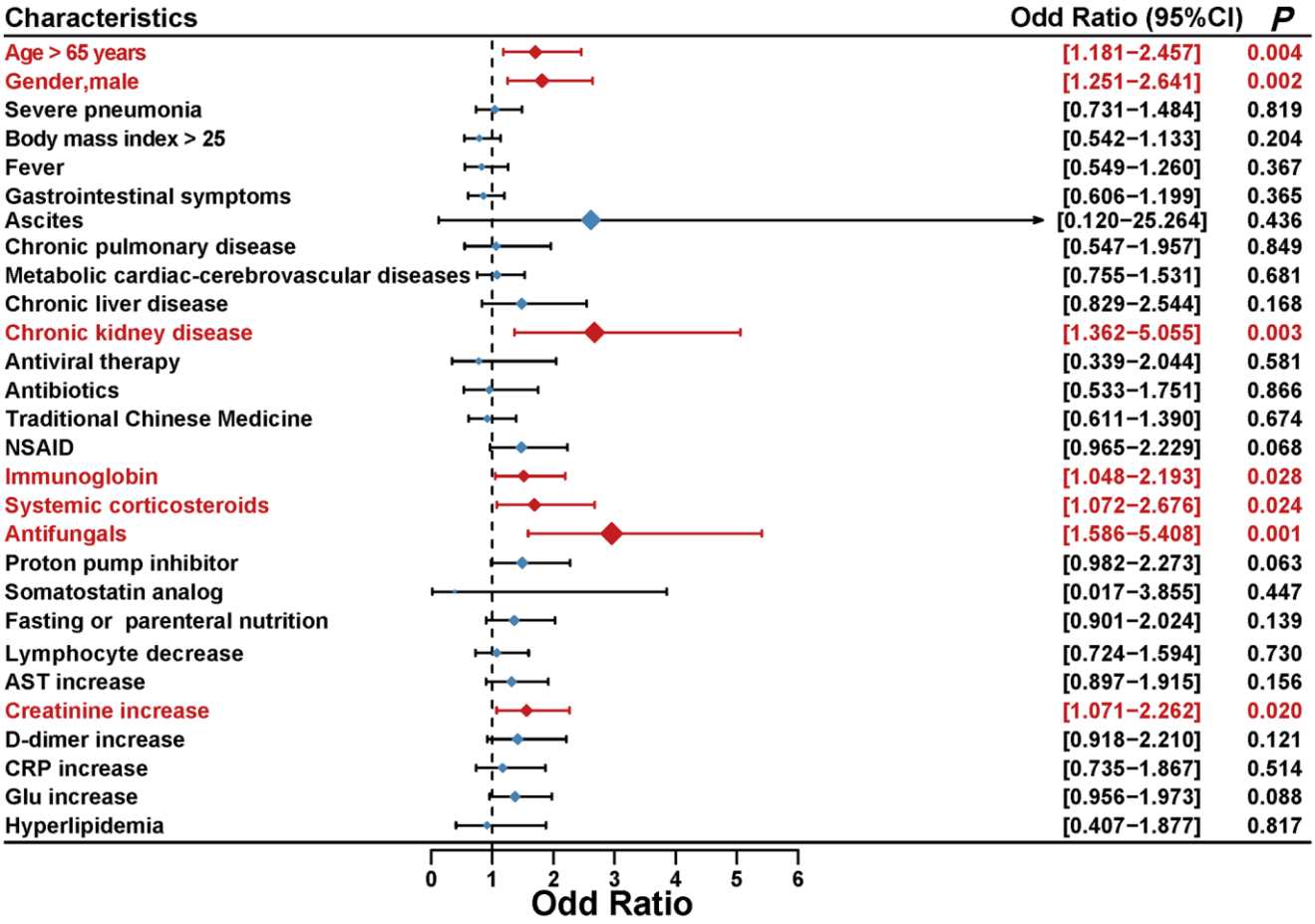

Figure 3. Multivariable ordered logistic regression analysis was performed to reveal the association between common clinical characteristics and peak amylase levels in COVID-19 patients.

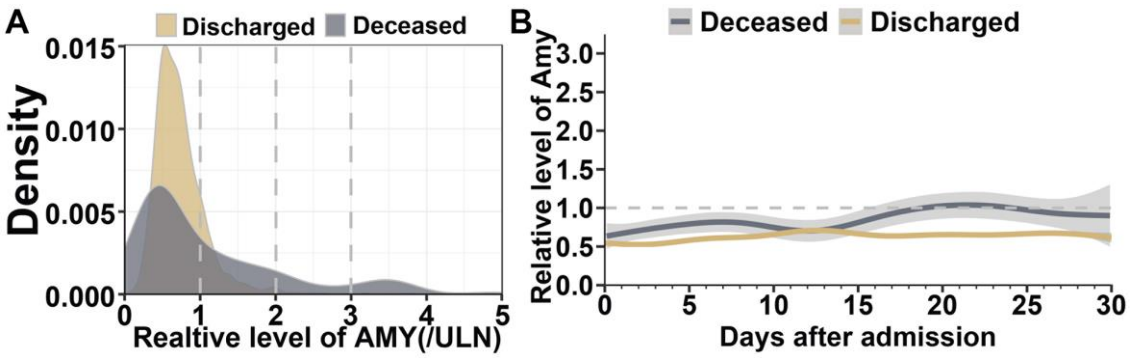

Figure 4. Dynamic profile of serum amylase in patients with COVID-19 pneumonia. (**A**) Kernel density estimates using Gaussian kernels to display serum amylase distributions between survived patients and those who died of COVID-19. (**B**) Smooth trajectories of mean amylase between survived and deceased patients with 95% confidence band based on GAM.

pancreatic injury. Nevertheless, it is cursory to concluded that SARS-COV-2 lead to mild or slight pancreatic injury, for higher serum levels of amylase can be caused by non-pancreatic etiologies, including intestinal disease [30], malignancy [30], acidosis [31], renal failure [32], and diabetes [32], other than pancreatic injury. On the other hand, a previous animal model indicated that epithelial cells lining salivary gland ducts were the early targets by the predecessor of SARS-CoV-2, SARS-CoV-1, which could contribute to serum amylase elevation other than pancreatic injury [33]. Besides, any physiological change, including elevated vascular permeability, disordered lymphatic drainage, and abnormal clearance would give rise to serum hyperamylasemia. Therefore, impaired renal excretion of amylase might be one of the major sources of hyperamylasemia in our cohort, which was similar to previous studies [34, 35].

The major COVID-19 treatment included antivirals, antibiotics, and systemic corticosteroids and antifungal was more likely to be administrated in weaker patients [36]. In our study, the results indicated that use of NSAIDs, immunoglobin, antifungal and systemic corticosteroids presented a positive associated with serum hyperamylasemia. Although these evidences could not directly infer the causal impact of drugs on hyperamylasemia, it is recommended that clinicians should pay more attention to the drug toxicity during the treatment of COVID-19 infection.

There is a discrepancy on the serum amylase testing between the health providers. Nevertheless, we discriminated no difference in the prevalence of gastrointestinal symptoms between patients with elevated serum and their counterparts of normal amylase level, which imply that they were not the primary factor that impelled clinicians to check the amylase in COVID-19 patients. Interestingly, our data seemed that gastrointestinal symptoms were not associated with serum amylase, but significantly more prevalent in individuals with amylase level more than 3 times ULN, the probability of hyperamylasemia resulted from colonic or enteric involvement of the virus as well as cannot be excluded. Further investigations including large number of patients with more than 3 times ULN amylase are needed to better understand the association between hyperamylasemia and gastrointestinal symptoms.

Although it was less specificity to diagnose pancreatic injury merely according to hyperamylasemia [16, 29], the probability of pancreatic damage cannot be eliminated. Due to high expression of angiotensin-converting enzyme-2 (*ACE2*) receptors and transmembrane serine protease 2 (*TMPRSS2*) in pancreatic tissue

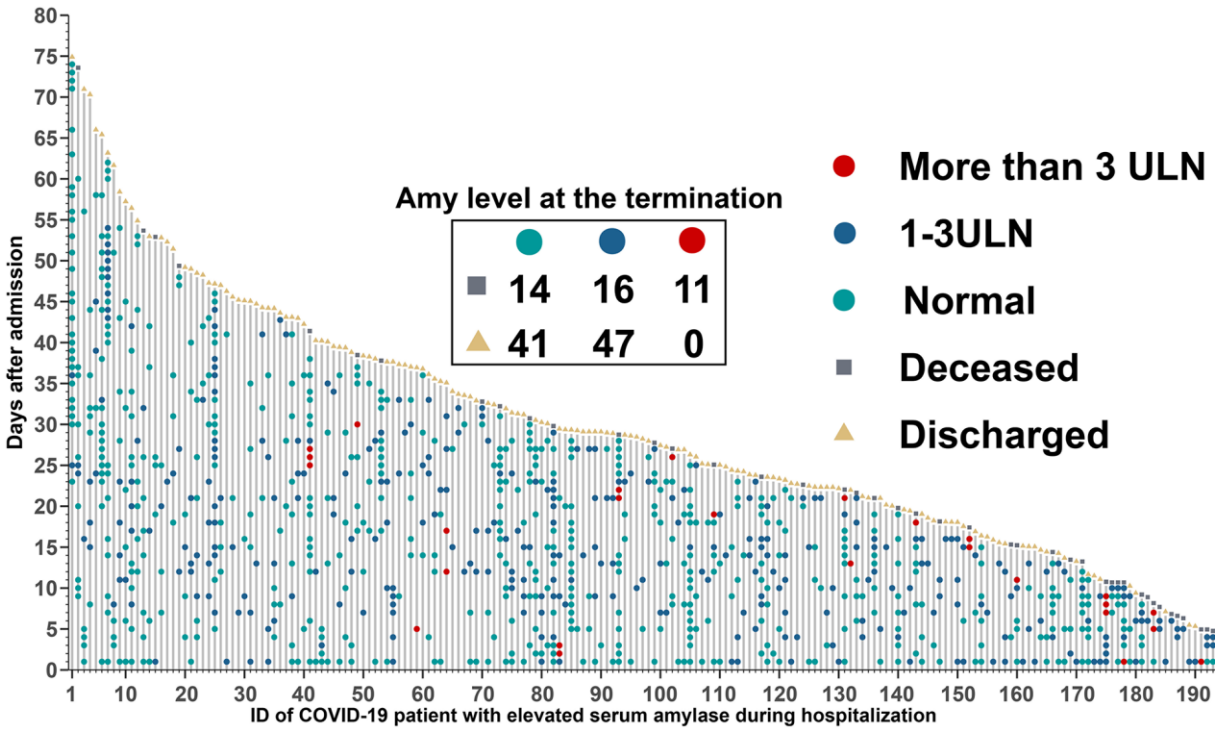

Figure 5. Timeline of events for 196 COVID-19 patients with hyperamylasemia. Amylase level at the termination time was extracted within 5 days before discharge or death, and 67 patients with no available data.

(Supplementary Figure 2), it is reasonable to suppose that the pancreas could be the attack target of SARS-CoV-2 [37, 38]. Moreover, this ratiocination was supported by a family cluster of acute pancreatitis associated with SARS-CoV-2 in Denmark [39], the detection of SARS-CoV2 RNA in a pancreatic pseudocyst fluid sample collected from a COVID-19 patient [40], autopsy specimen of COVID-19 patients who presented with slightly degeneration in pancreatic islet [41]. Hereby, whether the potential pancreatic injury caused by the direct attack by SARS-COV-2 or as part of the secondary hypoxemia, systemic inflammatory response and cytokine cascade leading to multi-organ damage. Nevertheless, clinical manifestations and limited radiological evidence in those who had a serum amylases above 3 ULN revealed that no diagnosis of acute pancreatitis could be established in our study. However, the probability of mild pancreatic injury that imaging examinations might not be discriminated could not be excluded. Consequently, serum amylase level elevation in COVID19 may be attributed to pancreatic injury, multiorgan-damaged involvement, as well as drug toxicity or multi-factor. Though, our results show that elevated amylase might be an independent risk factor of detrimental outcomes in COVID-19 infection.

Our study suffered several limitations. Firstly, due to its retrospective nature, the multiple tests for serum amylase were performed at different time intervals for each individual. Hence, bias might occur on the condition that when a patient's condition deteriorates, more tests are done and usually get a worse result than in patients with a better course of illness. secondly, given the observational nature of our study, it could only demonstrate association, not causation. Whether hyperamylasemia is caused by SARS-CoV-2 needs to be further evaluated by direct clinical investigation. Last but not least, no measures were taken to distinguish analytically the distinct isoforms of serum amylase, so we could not reveal the real source of amylase (salivary or pancreatic amylase).

CONCLUSIONS

The dynamic patterns of serum amylase and their potential risk factors might provide a crucial explanation for the COVID-19-related hyperamylasemia. Since hyperamylasemia is significantly associated with in-hospital mortality, it is crucial to monitor serum amylase vigilantly in COVID-19 patients.

AUTHOR CONTRIBUTIONS

All authors were responsible for the study concept and design. BZ, XC, and DZ were responsible for the acquisition and analysis of data, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors were responsible for the interpretation of data, the drafting and critical revision of the manuscript for important intellectual content.

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CONFLICTS OF INTEREST

The authors declare no conflicts of financial interest.

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REFERENCES

1. Williamson EJ, Walker AJ, Bhaskaran K, Bacon S, Bates C, Morton CE, Curtis HJ, Mehrkar A, Evans D, Inglesby P, Cockburn J, McDonald HI, MacKenna B, et al. Factors associated with COVID-19-related death using OpenSAFELY. Nature. 2020; 584:430–36. https://doi.org/10.1038/s41586-020-2521-4 PMID:32640463

2. Jin X, Lian JS, Hu JH, Gao J, Zheng L, Zhang YM, Hao SR, Jia HY, Cai H, Zhang XL, Yu GD, Xu KJ, Wang XY, et al. Epidemiological, clinical and virological characteristics of 74 cases of coronavirus-infected disease 2019 (COVID-19) with gastrointestinal symptoms. Gut. 2020; 69:1002–09. https://doi.org/10.1136/gutjnl-2020-320926 PMID:32213556

3. Guo T, Fan Y, Chen M, Wu X, Zhang L, He T, Wang H, Wan J, Wang X, Lu Z. Cardiovascular Implications of Fatal Outcomes of Patients With Coronavirus Disease 2019 (COVID-19). JAMA Cardiol. 2020; 5:811–18. https://doi.org/10.1001/jamacardio.2020.1017 PMID:32219356

4. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, et al. Clinical characteristics of 113 deceased patients with

coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091. https://doi.org/10.1136/bmj.m1091 PMID:32217556

5. Grasselli G, Zangrillo A, Zanella A, Antonelli M, Cabrini L, Castelli A, Cereda D, Coluccello A, Foti G, Fumagalli R, Iotti G, Latronico N, Lorini L, et al, and COVID-19 Lombardy ICU Network. Baseline Characteristics and Outcomes of 1591 Patients Infected With SARS-CoV-2 Admitted to ICUs of the Lombardy Region, Italy. JAMA. 2020; 323:1574–81. https://doi.org/10.1001/jama.2020.5394 PMID:32250385

6. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell L, Chernyak Y, Tobin KA, Cerfolio RJ, Francois F, Horwitz LI. Factors associated with hospital admission and critical illness among 5279 people with coronavirus disease 2019 in New York City: prospective cohort study. BMJ. 2020; 369:m1966. https://doi.org/10.1136/bmj.m1966 PMID:32444366

7. Wu C, Chen X, Cai Y, Xia J, Zhou X, Xu S, Huang H, Zhang L, Zhou X, Du C, Zhang Y, Song J, Wang S, et al. Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. JAMA Intern Med. 2020; 180:934–43. https://doi.org/10.1001/jamainternmed.2020.0994 PMID:32167524

8. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet. 2020; 395:1054–62. https://doi.org/10.1016/S0140-6736(20)30566-3 PMID:32171076

9. Zhang P, Zhu L, Cai J, Lei F, Qin JJ, Xie J, Liu YM, Zhao YC, Huang X, Lin L, Xia M, Chen MM, Cheng X, et al. Association of Inpatient Use of Angiotensin-Converting Enzyme Inhibitors and Angiotensin II Receptor Blockers With Mortality Among Patients With Hypertension Hospitalized With COVID-19. Circ Res. 2020; 126:1671–81. https://doi.org/10.1161/CIRCRESAHA.120.317134 PMID:32302265

10. Ding ZY, Li GX, Chen L, Shu C, Song J, Wang W, Wang YW, Chen Q, Jin GN, Liu TT, Liang JN, Zhu P, Zhu W, et al, and Tongji Multidisciplinary Team for Treating COVID-19 (TTTC). Association of liver abnormalities with in-hospital mortality in patients with COVID-19. J Hepatol. 2021; 74:1295–302. https://doi.org/10.1016/j.jhep.2020.12.012 PMID:33347952

11. Wang F, Wang H, Fan J, Zhang Y, Wang H, Zhao Q. Pancreatic Injury Patterns in Patients With Coronavirus Disease 19 Pneumonia. Gastroenterology. 2020; 159:367–70. https://doi.org/10.1053/j.gastro.2020.03.055 PMID:32247022

12. Barbieri JS, Riggio JM, Jaffe R. Amylase testing for abdominal pain and suspected acute pancreatitis. J Hosp Med. 2016; 11:366–68. https://doi.org/10.1002/jhm.2544 PMID:27160507

13. Huang C, Bai L, Xue X, Peng L, Jiang J, Zhang X. Hyperamylasemia as an early predictor of mortality in patients with acute paraquat poisoning. J Int Med Res. 2020; 48:300060520910037. https://doi.org/10.1177/0300060520910037 PMID:32223576

14. Tositti G, Fabris P, Barnes E, Furlan F, Franzetti M, Stecca C, Pignattari E, Pesavento V, de Lalla F. Pancreatic hyperamylasemia during acute gastroenteritis: incidence and clinical relevance. BMC Infect Dis. 2001; 1:18. https://doi.org/10.1186/1471-2334-1-18 PMID:11667952

15. Azzopardi E, Lloyd C, Teixeira SR, Conlan RS, Whitaker IS. Clinical applications of amylase: Novel perspectives. Surgery. 2016; 160:26–37. https://doi.org/10.1016/j.surg.2016.01.005 PMID:27117578

16. de-Madaria E, Siau K, Cárdenas-Jaén K. Increased Amylase and Lipase in Patients With COVID-19 Pneumonia: Don't Blame the Pancreas Just Yet! Gastroenterology. 2021; 160:1871. https://doi.org/10.1053/j.gastro.2020.04.044 PMID:32330475

17. McNabb-Baltar J, Jin DX, Grover AS, Redd WD, Zhou JC, Hathorn KE, McCarty TR, Bazarbashi AN, Shen L, Chan WW. Lipase Elevation in Patients With COVID-19. Am J Gastroenterol. 2020; 115:1286–88. https://doi.org/10.14309/ajg.0000000000000732 PMID:32496339

18. Du YQ, Chen QK, Li HY, Zeng Y. Chinese guidelines for the management of acute pancreatitis (Shenyang, 2019). J Clin Hepatol. 2019; 35:2706–11.

19. Gando S, Wada H, Thachil J, and Scientific and Standardization Committee on DIC of the International Society on Thrombosis and Haemostasis (ISTH). Differentiating disseminated intravascular coagulation (DIC) with the fibrinolytic phenotype from coagulopathy of trauma and acute coagulopathy of trauma-shock (COT/ACOTS). J Thromb Haemost. 2013; 11:826–35. https://doi.org/10.1111/jth.12190 PMID:23522358

20. Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, Chapman MJ, De Backer GG, Delgado V, Ference BA, Graham IM, Halliday A, Landmesser U, et

al, and ESC Scientific Document Group. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. Eur Heart J. 2020; 41:111–88. https://doi.org/10.1093/eurheartj/ehz455 PMID:31504418

21. Goldman MJ, Craft B, Hastie M, Repečka K, McDade F, Kamath A, Banerjee A, Luo Y, Rogers D, Brooks AN, Zhu J, Haussler D. Visualizing and interpreting cancer genomics data via the Xena platform. Nat Biotechnol. 2020; 38:675–78. https://doi.org/10.1038/s41587-020-0546-8 PMID:32444850

22. Vivian J, Rao AA, Nothaft FA, Ketchum C, Armstrong J, Novak A, Pfeil J, Narkizian J, Deran AD, Musselman-Brown A, Schmidt H, Amstutz P, Craft B, et al. Toil enables reproducible, open source, big biomedical data analyses. Nat Biotechnol. 2017; 35:314–16. https://doi.org/10.1038/nbt.3772 PMID:28398314

23. Wood SN. Stable and efficient multiple smoothing parameter estimation for generalized additive models. J Am Stat Assoc. 2004; 99:673–86. https://doi.org/10.1198/016214504000000980

24. Ripatti S, Palmgren J. Estimation of multivariate frailty models using penalized partial likelihood. Biometrics. 2000; 56:1016–22. https://doi.org/10.1111/j.0006-341x.2000.01016.x PMID:11129456

25. Bates D, Machler M, Bolker BM, Walker SC. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015; 67:1–48. https://doi.org/10.18637/jss.v067.i01

26. Bland JM, Altman DG. Survival probabilities (the Kaplan-Meier method). BMJ. 1998; 317:1572. https://doi.org/10.1136/bmj.317.7172.1572 PMID:9836663

27. Desquilbet L, Mariotti F. Dose-response analyses using restricted cubic spline functions in public health research. Stat Med. 2010; 29:1037–57. https://doi.org/10.1002/sim.3841 PMID:20087875

28. Andersson T, Alfredsson L, Källberg H, Zdravkovic S, Ahlbom A. Calculating measures of biological interaction. Eur J Epidemiol. 2005; 20:575–79. https://doi.org/10.1007/s10654-005-7835-x PMID:16119429

29. Banks PA, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS, and Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. Gut. 2013; 62:102–11. https://doi.org/10.1136/gutjnl-2012-302779 PMID:23100216

30. Pieper-Bigelow C, Strocchi A, Levitt MD. Where does serum amylase come from and where does it go? Gastroenterol Clin North Am. 1990; 19:793–810. https://doi.org/10.1016/S0889-8553(21)00514-8 PMID:1702756

31. Shi S, Qin M, Shen B, Cai Y, Liu T, Yang F, Gong W, Liu X, Liang J, Zhao Q, Huang H, Yang B, Huang C. Association of Cardiac Injury With Mortality in Hospitalized Patients With COVID-19 in Wuhan, China. JAMA Cardiol. 2020; 5:802–10. https://doi.org/10.1001/jamacardio.2020.0950 PMID:32211816

32. Hameed AM, Lam VW, Pleass HC. Significant elevations of serum lipase not caused by pancreatitis: a systematic review. HPB (Oxford). 2015; 17:99–112. https://doi.org/10.1111/hpb.12277 PMID:24888393

33. Liu L, Wei Q, Alvarez X, Wang H, Du Y, Zhu H, Jiang H, Zhou J, Lam P, Zhang L, Lackner A, Qin C, Chen Z. Epithelial cells lining salivary gland ducts are early target cells of severe acute respiratory syndrome coronavirus infection in the upper respiratory tracts of rhesus macaques. J Virol. 2011; 85:4025–30. https://doi.org/10.1128/JVI.02292-10 PMID:21289121

34. Seno T, Harada H, Ochi K, Tanaka J, Matsumoto S, Choudhury R, Mizushima T, Tsuboi K, Ishida M. Serum levels of six pancreatic enzymes as related to the degree of renal dysfunction. Am J Gastroenterol. 1995; 90:2002–05. PMID:7485010

35. Paajanen H, Nuutinen P, Harmoinen A, Pöyhönen M, Pitkänen O, Nordback I, Grönroos J, Nevalainen TJ. Hyperamylasemia after cardiopulmonary bypass: pancreatic cellular injury or impaired renal excretion of amylase? Surgery. 1998; 123:504–10. https://doi.org/10.1067/msy.1998.88093 PMID:9591002

36. Qiu H, Tong Z, Ma P, Hu M, Peng Z, Wu W, Du B, and China Critical Care Clinical Trials Group (CCCCTG). Intensive care during the coronavirus epidemic. Intensive Care Med. 2020; 46:576–78. https://doi.org/10.1007/s00134-020-05966-y PMID:32077996

37. Wong SH, Lui RN, Sung JJ. Covid-19 and the digestive system. J Gastroenterol Hepatol. 2020; 35:744–48. https://doi.org/10.1111/jgh.15047 PMID:32215956

38. Tian S, Hu N, Lou J, Chen K, Kang X, Xiang Z, Chen H, Wang D, Liu N, Liu D, Chen G, Zhang Y, Li D, et al. Characteristics of COVID-19 infection in Beijing. J Infect. 2020; 80:401–06.

https://doi.org/10.1016/j.jinf.2020.02.018 PMID:32112886

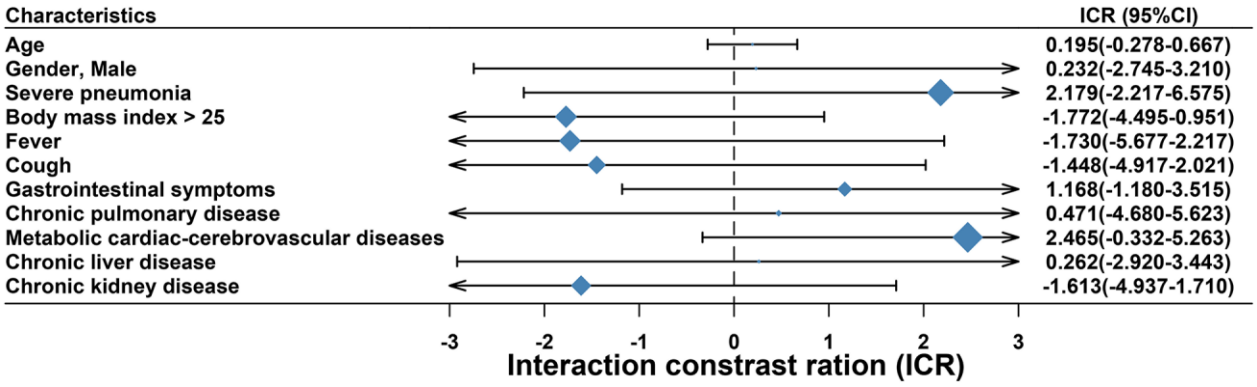
39. Hadi A, Werge M, Kristiansen KT, Pedersen UG, Karstensen JG, Novovic S, Gluud LL. Coronavirus Disease-19 (COVID-19) associated with severe acute pancreatitis: Case report on three family members. Pancreatology. 2020; 20:665–67. https://doi.org/10.1016/j.pan.2020.04.021 PMID:32387082

40. Schepis T, Larghi A, Papa A, Miele L, Panzuto F, De Biase L, Annibale B, Cattani P, Rapaccini GL. SARS-CoV2 RNA detection in a pancreatic pseudocyst sample. Pancreatology. 2020; 20:1011–12. https://doi.org/10.1016/j.pan.2020.05.016 PMID:32498972

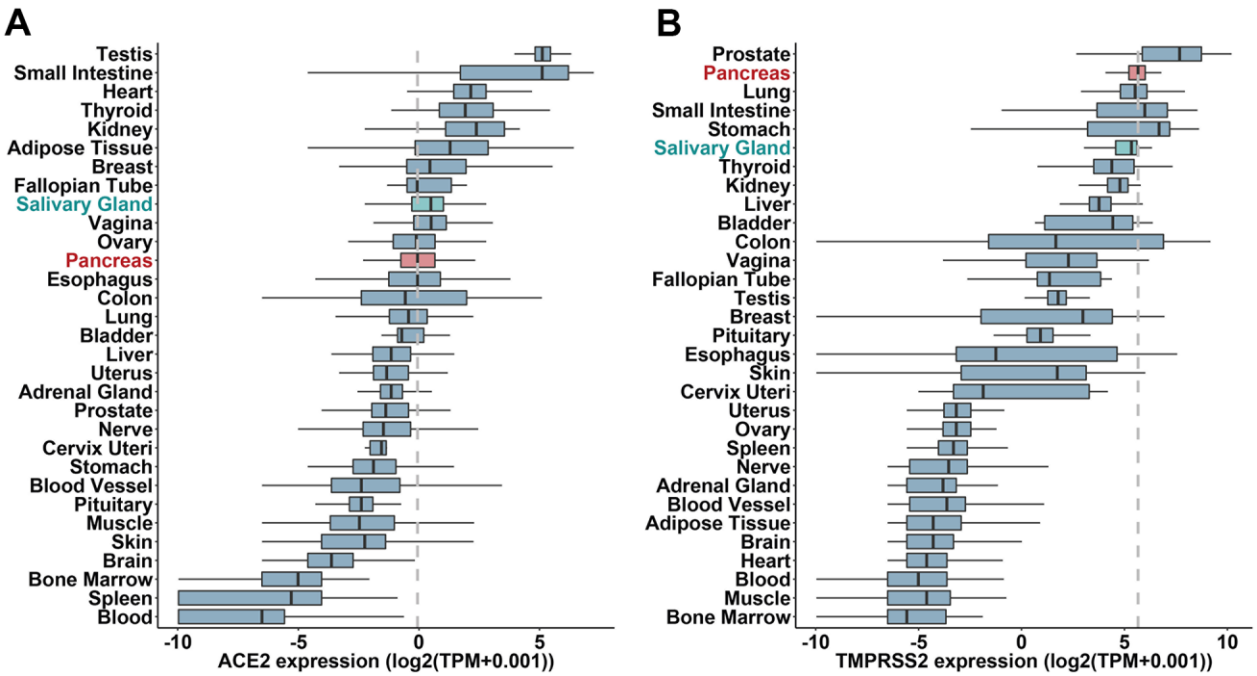
41. Yao XH, Li TY, He ZC, Ping YF, Liu HW, Yu SC, Mou HM, Wang LH, Zhang HR, Fu WJ, Luo T, Liu F, Guo QN, et al. [A pathological report of three COVID-19 cases by minimal invasive autopsies]. Zhonghua Bing Li Xue Za Zhi. 2020; 49:411–17. https://doi.org/10.3760/cma.j.cn112151-20200312-00193 PMID:32172546

SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. Interaction contrast ratios (ICR) were calculated to assess the additive interaction between serum amylase and common clinical characteristics in the Cox regression.



Supplementary Figure 2. The mRNA expression of *ACE2* (**A**) and *TMPRSS2* (**B**) in 31 normal human tissues from GTEx.

Supplementary Table

Supplementary Table 1. The clinical characteristics of patients with 3 times ULN.

Characteristics	Above 3 folds of ULN (N=19)
Age- yr	69[64, 76]
Age≥ 65	14 (73.7)
Male	11 (57.9)
BMI	23.87 [23.47, 25.74]
Time from illness onset to hospital admission, days	10[7, 15]
Severe pneumonia*	14 (73.7)
Signs and symptoms	
Fever	13 (68.4)
Cough	14 (73.7)
Fatigue	4 (21.1)
Chest pain	1 (5.3)
Gastrointestinal symptoms**	10 (52.6)
Dyspnea	9 (47.4)
Myalgia	1 (5.3)
Administration of mechanical ventilation	15 (78.9)
Non-invasive	1 (5.3)
Invasive	14 (73.7)
Admission to intensive care unit	16 (84.2)
Renal replacement therapy	7 (36.8)
Extracorporeal membrane oxygenation	1 (5.3)
Clinical outcomes	
In-hospital death	15 (78.9)
Duration from illness onset to death, days	31[23, 36]
Hospital discharge	4 (21.1)
Duration from illness onset to discharge, days	41[37, 46]
Complications	
Acute kidney injury	13 (68.4)
Acute respiratory distress syndrome	15 (78.9)
Acute heart failure	16 (88.9)
Cardiac injury	16 (88.9)
Sepsis	15 (78.9)
Disseminated intravascular coagulation	11 (57.9)

1. Data were provided as number (percentage), median (interquartile range).
2. Serum Amylase Level, in a healthy individual, a normal blood amylase level ranges from 0-115 units per liter (U/L) in our hospital. Patients with serum amylase >345 U/L were classified into group of above 3 Folds of ULN (N=19).
3. Severe pneumonia*, the illness severity was classified according to Guidance for Corona Virus Disease 2019 (6/7th edition) released by the National Health Commission of China; Gastrointestinal symptoms**, including anorexia, nausea or vomiting, diarrhea, abdominal pain.

Research Paper

Multicenter study evaluating one multiplex RT-PCR assay to detect SARS-CoV-2, influenza A/B, and respiratory syncytia virus using the LabTurbo AIO open platform: epidemiological features, automated sample-to-result, and high-throughput testing

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ABSTRACT

Since the Coronavirus 19 (COVID-19) pandemic, several SARS-CoV-2 variants of concern (SARS-CoV-2 VOC) have been reported. The B.1.1.7 variant has been associated with increased mortality and transmission risk. Furthermore, cluster and possible co-infection cases could occur in the next influenza season or COVID-19 pandemic wave, warranting efficient diagnosis and treatment decision making. Here, we aimed to detect SARS-CoV-2 and other common respiratory viruses using multiplex RT-PCR developed on the LabTurbo AIO 48 open system. We performed a multicenter study to evaluate the performance and analytical sensitivity of the LabTurbo AIO 48 system for SARS-CoV-2, influenza A/B, and respiratory syncytial virus (RSV) using 652 nasopharyngeal swab clinical samples from patients. The LabTurbo AIO 48 system demonstrated a sensitivity of 9.4 copies/per PCR for *N2* of SARS-CoV-2; 24 copies/per PCR for *M* of influenza A and B; and 24 copies/per PCR for *N* of RSV. The assay presented consistent performance in the multicenter study. The multiplex RT-PCR applied on the LabTurbo AIO 48 open platform provided highly sensitive, robust, and accurate results and enabled high-throughput detection of B.1.1.7, influenza A/B, and RSV with short turnaround times. Therefore, this automated molecular diagnostic assay could enable streamlined testing if COVID-19 becomes a seasonal disease.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), has spread worldwide, with over 180 million confirmed cases of infection (https://covid19.who.int/ accessed: 2021/07/06). The B.1.1.7 variant is estimated to have emerged in September 2020 and has quickly become the dominant circulating SARS-CoV-2 variant in England [1, 2]. Taiwan faced the third outbreak of COVID-19 just before the third week of April and is still ongoing. This third outbreak involved the alpha variant (B.1.1.7) [3, 4].

Currently, real-time reverse transcription-polymerase chain reaction (rRT-PCR) is the gold standard assay for early diagnosis in patients with suspected SARS-CoV-2 infection [5–7]. However, some studies have showed that targeting a particular detection region might result in the loss of sensitivity for various SARS-CoV-2 variants [8]. In addition, it is sometimes difficult to distinguish COVID-19 from other respiratory illnesses caused by other respiratory viruses because of common clinical manifestations including fever, cough, and dyspnea [9–12]. Consequently, SARS-CoV-2 and other upper respiratory viruses, including influenza virus and respiratory syncytial virus (RSV), have to be concurrently tested by rRT-PCR in symptomatic patients.

Several commercial kits support the rapid detection of multiple pathogens, including SARS-CoV-2, influenza A/B, and other respiratory pathogens [13]. However, these kits were not affordable cost and could not offer high throughput during the COVID-19 pandemic in developed countries. In addition, in light of the high demand for nucleic acid-amplification tests, continuing shortage of supplies, and high sensitivity of molecular diagnostics, there is a need for one multiplex assay to simultaneously screen all four viruses (SARS-CoV-2, influenza A, B, and RSV) with the same reaction [14].

In this study, we developed a sample-to-result platform that fully automated laboratory-developed multiplex RT-PCR assay to simultaneously detect SARS-CoV-2, influenza A/B, and RSV in one tube on the LabTurbo AIO 48 system.

RESULTS

Epidemiological features

We retrieved the clinical dataset of 102 patients infected by the SARS-CoV-2 B.1.1.7 variant. We analyzed RNA extracted from positive specimens using VirSNiP SARS-CoV-2 Spike N501Y and Spike del H69/V70 (TIB Molbiol, Berlin, Germany) to confirm the variant type. All data of all patients were reported between May 1 and July 4, 2021. We analyzed age distribution of the SARS-CoV-2 (B.1.1.7)-positive patients in our dataset (Table 1) and found that the youngest and oldest non-survivors died at 43 and 91 years of age, respectively. Elderly patients ≥70 years accounted for only 59% of the 22 survivors. Our sex analysis showed that COVID-19-related deaths were more among elderly males (50%, total 14) than among younger males (15%, total 40). Female patients showed a similar proportion of non-survivors, with mortality of 30.0% in elderly females and 5.3% in younger females (Table 2).

Analytical sensitivity of the one multiplex rRT-PCR on the LabTurbo AIO 48 open platform

To validate the sensitivity of the designed multiplex, we tested all these pathogens (SARS-CoV-2, influenza A virus (subtypes H1, H1N1, and H3), influenza B virus, and RSV (subtypes A and B)) in one multiplex RT-PCR on the AIO48 open system. We tested several dilutions to determine the LoD by repeating the experiments 20 times. We defined limit of detection (LoD) as the minimum concentration with a positive detection rate of 95%. The LoD for each target was 9.4 copies/PCR reaction for the *N2* gene of SARS-CoV-2; the LoD for influenza A/B virus (*M* gene) and RSV (*N* gene) reached 24 copies/PCR (Table 3). A mixed RNA sample was also tested using the same protocol. The LoD was the same as the above (Supplementary Table 1). Additionally, there was no cross-reaction among the respiratory pathogens.

Analytical specificity of the one multiplex rRT-PCR on the LabTurbo AIO 48 platform

We tested the analytical specificity of the lab-developed multiplex PCR test performed on the LabTurbo AIO 48 system for upper respiratory viruses other than SARS-CoV-2, influenza A/B virus, and RSV. Clinical samples or cell supernatants positive for rhinovirus/enterovirus, parainfluenza virus, cytomegalovirus, herpes simplex virus, varicella-zoster virus, and adenovirus were obtained from the Taiwan CDC Viral Infection Contract Laboratory. There was no cross-reactivity among these organisms (Table 4).

Clinical performance of the multiplex rRT-PCR on the LabTurbo AIO 48 system platform

We analyzed 652 retrospective specimens in this study: 102 from SARS-CoV-2-positive patients and 550 from SARS-CoV-2-negative patients. The *N2* gene from the multiplex rRT-PCR mixture and *RdRp* and *E* genes

Table 1. Clinical features of COVID-19 patients with the symptoms and clinical outcomes.

Characteristics	SARS-CoV-2 (B.1.1.7)
Total number	102
Gender	
Male	54
Female	48
Age	
<19	2
20–49	28
50–69	47
>70	25
Mean	62.6
Medium	64
Symptoms	
Fever	76
Cough	83
Difficulty breathing	30
Burnout	7
Diarrhea	17
Clinical outcome	
Survivors	84
Non-survivors	18

recommended by the WHO guidelines were compared for all positive specimens. Figure 1 shows a high correlation between the *N2* and *Rdrp* genes ($R^2 = 0.95$). Similar results were obtained for the *N2* and *E* genes ($R^2 = 0.95$). There was no apparent difference between those targets and no false positive nor negative result between those positive samples. Among the positive specimens, 102 specimens from SARS-CoV-2-positive patients were tested at two medical centers (Tri-Service General Hospital (TSGH) and Cathay General Hospital (CGH)) independently, followed by the same multiplex on the LabTurbo AIO platform. Those selected 102 samples with the Ct level that satisfied the range for high to low SARS-CoV-2 loads. Figure 2 shows our assay targeting the *N2* gene of SARS-CoV-2 without a significant difference in the results between the two medical centers. Furthermore, there were no false-positives or false-negatives among these specimens. The results of the two centers presented 100% agreement with each other.

Results of co-infection of SARS-CoV-2 and other respiratory pathogens

We analyzed 652 specimens tested for SARS-CoV-2 and other respiratory pathogens using our multiplex rRT-PCR on the AIO LabTurbo open platform. The 102 SARS-CoV-2-positive specimens were found to be positive for SARS-CoV-2, whereas none was found to be positive for one or more non–SARS-CoV-2 pathogen(s). Among the tested specimens, influenza A virus was the most commonly detected pathogen (n = 19), followed by influenza B virus (n = 5) and RSV (n = 10). This finding highlighted the importance of differentiating other causes of respiratory illness from SARS-CoV-2.

DISCUSSION

The SARS-CoV-2 B.1.1.7 variant of concern (VOC), which was first detected in South-East England, is more transmissible than previously circulating variants [15]. Currently, it accounts for 50–90% of the COVID-19 cases in the US and Europe and spreading over 170 countries [16]. During the COVID-19 pandemic, SARS-CoV-2 B.1.1.7 has been associated with increased secondary attack rate [17], and risk of hospitalization, severity, and mortality [18, 19]. In the present study, we used specimens from 102 individuals with COVID-19 between May 2021 and July 2021. Non-Survivors comprised 50% of the total elder males (>70 years old) and 30% of the total elderly females. Currently, Taiwan is facing the third wave of SARS-CoV-2 B.1.1.7 infection.

Our multiplex RT-PCR assay on the LabTurbo AIO 48 open platform showed good performance. COVID-19 symptoms are similar to flu-like symptoms. The flu-like

symptoms include fever, chills, headache, muscle or body aches, cough, sore throat, runny nose, fatigue, nausea, vomiting, and diarrhea, and they are caused by different respiratory tract pathogens. The major respiratory tract pathogens include influenza A and B, RSV, adenovirus, enterovirus, human metapneumovirus, parainfluenza virus, adenovirus, rhinovirus, and human coronavirus. Hence, the pathogens causing these symptoms will be difficult to distinguish in the next flu season [20]. The death rate associated with different viruses varies worldwide. The death rate of patients infected with influenza virus could reach 250,000–500,000 individuals worldwide. RSV is associated with an estimated 132,000–172,000 pediatric hospitalizations in the United States annually. Since 2020, SARS-CoV-2 infection has resulted in over four million deaths. In addition, 3% of patients with COVID-19 are co-infected by other respiratory tract viruses. Influenza A virus and RSV were the top two pathogens, which accounted for 30% of viral co-infections [21]. Recently, several companies have developed commercial kits to detect SARS-CoV-2, influenza virus, and/or RSV, such as Liat SARS-CoV-2 and Influenza A/B (Roche Molecular Systems, Inc., Pleasanton, CA, USA), Cepheid Xpert Xpress SARS-CoV-2/Flu/RSV (Cepheid, Sunnyvale, CA, USA), and BioFire Respiratory Panel 2.1 (RP2.1; BioFire Diagnostics, LLC, Salt Lake City, UT, USA). However, these molecular diagnostic tests rely on specific platforms with specific reagent requirements. That turnaround time of these commercial kit is approximately 25–45 min for one sample. The turnaround time increases with the number of specimens. For example, in BioFire Respiratory Panel 2.1, 48 specimens can be tested in 36 h with one machine. The capacity of the multiplex RT-PCR assay that we developed could reach approximately 2 h for 48 samples. It will decrease the TAT by three-fold for 48 samples. Thus, our assay is a

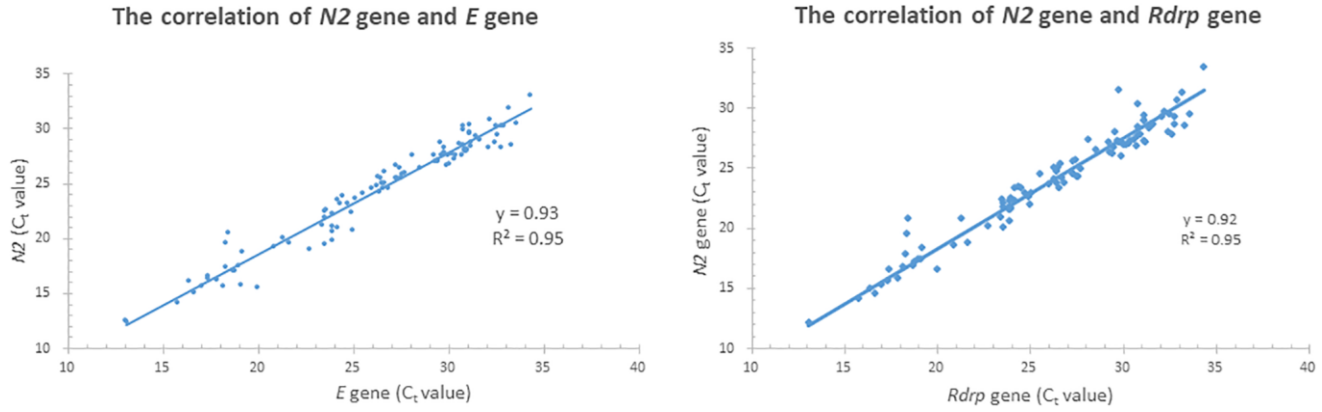

Figure 1. The correlation between *N2*, *E* and *Rdrp* gene of 102 SARS-CoV-2 positive specimens.

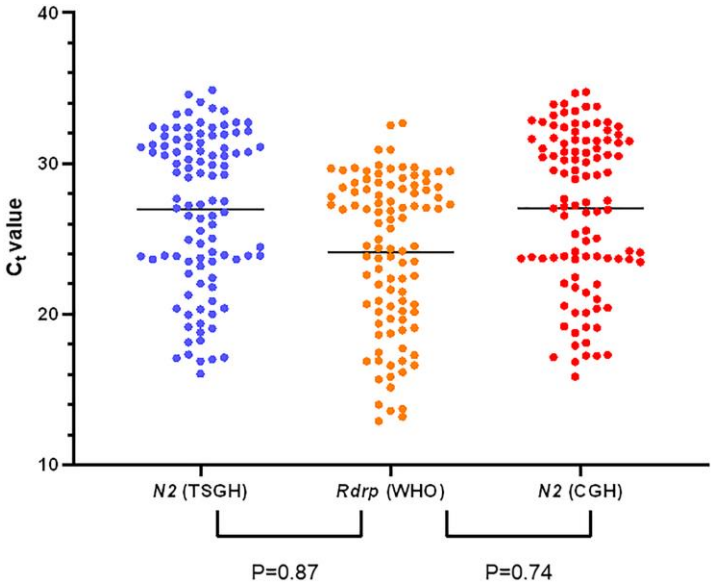

Figure 2. The clinical performance of multiplex RT-PCR in SARS-CoV-2 positive specimens between the two medical centers.

Table 2. Basic information of 102 SARS-CoV-2 (B.1.1.7) patients in our study.

	Age	Survivors	Non-Survivors
Male			
	Younger (<70)	34 (85.0%)	6 (15.0%)
	Elder (>70)	7 (50.0%)	7 (50.0%)
Female			
	Younger (<70)	36 (94.7%)	2 (5.3%)
	Elder (>70)	7 (70.0%)	3 (30.0%)
Total		84	18

Table 3. Assessment of Limit of detection for SARS-CoV-2, influenza A/B, RSV in multiplex RT-PCR.

Pathogen	Gene target/fluorescent dye	No. of replicates detected at each dilution/total no. of replicates at indicated no. of copies per PCR (percentage)				
		300	75	24	18.8	9.4
SARS-CoV-2	*N2*/FAM	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)
Influenza A H1	*M*/VIC	20/20 (100)	20/20 (100)	20/20 (100)	16/20 (80)	N.D.
Influenza A H3		20/20 (100)	20/20 (100)	20/20 (100)	9/20 (45)	N.D.
Influenza A H1N1		20/20 (100)	20/20 (100)	20/20 (100)	16/20 (80)	N.D.
Influenza B	*M*/Cy5	20/20 (100)	20/20 (100)	20/20 (100)	10/20 (50)	N.D.
RSV subtype A	*N*/Cy5.5	20/20 (100)	20/20 (100)	20/20 (100)	12/20 (60)	N.D.
RSV subtype B		20/20 (100)	20/20 (100)	20/20 (100)	8/20 (40)	N.D.

N.D. is not detected.

Table 4. The cross-reactivity tests of multiple RT-PCR from clinical samples or cell supernatants.

Pathogen	Gene targets of multiple rRT-PCR (positive no./total test no.)			Final performance
	N2 gene	*M* gene	*N* gene	
Parainfluenza virus	0/3	0/3	0/3	N.D.
Rhinovirus/Enterovirus	0/2	0/2	0/2	N.D.
Varicella-Zoster Virus	0/5	0/5	0/5	N.D.
Cytomegalovirus	0/5	0/5	0/5	N.D.
Herpes simplex virus type1 type 1	0/5	0/5	0/5	N.D.
Adenovirus	0/5	0/5	0/5	N.D.

N.D. is not detected.

valuable tool with a better efficacy and turnaround time for the simultaneous detection of SARS-CoV-2, influenza A virus, influenza B virus, and RSV than commercial kits. Our study provides a perspective to decide which molecular diagnostic test to implement in clinical laboratories. Our assay could accurately identify SARS-CoV-2 and other common respiratory viral infections. Simultaneous testing of all four pathogens shortens the turnaround time and could thus increase the effectiveness of control and prevention measures by health providers and departments. Infections with common respiratory viruses are associated with similar symptoms that are not easy to distinguish from each other. Furthermore, in patients with COVID-19, a pooled proportion meta-analysis has shown that 3% of patients were co-infected with other viruses [21]. Hence, in the next flu season, the multiplex RT-PCR might be a valuable tool to distinguish pathogens.

To diagnose RNA virus infections, RT-PCR is the most common method owing to its accuracy and popularity [22]. Currently, numerous primers have been designed to target various RNA sequences in six genes of SARS-CoV-2 for diagnostic purposes, including *ORF*1a/b, *RdRp* (RNA-dependent RNA polymerase), *S* (spike protein), *E* (envelope), and

N1/N2/N3 (nucleocapsid). Among these, the nucleocapsid *N2* and envelope *E* genes can be most sensitively detected, as described previously [23]. Here, we targeted the *N2* gene of SARS-CoV-2 and demonstrated that the performance of our method in detecting the *N2* gene was as good as that for detecting the *E* and *RdRp* genes, which are targeted by the WHO protocol. There was no apparent difference between the two targets and there is no false positive nor negative result between those positive samples. Hence, this multiplex could afford a good performance in preventing the spread of COVID-19.

However, our study has some limitations. First, the number of positive cases was small, as the number of initially confirmed COVID-19 cases in Taiwan was approximately 1,000. Thus, studies with a higher number of positive cases are required in the future. Furthermore, to assess the clinical performance of the LabTurbo AIO 48 system in detecting common upper respiratory viral pathogens, including SARS-CoV-2, we enrolled two medical centers. Our concern regarding this approach was whether the initial challenges encountered during the management of patients with COVID-19 potentially decreased the number of requests for virus culture tests to rule out other infections. Nevertheless, we used different instruments from the two medical centers to verify the same specimen, with consistent results. Second, this multiplex reagent just provides one *N2* gene for SARS-CoV-2. This was a screen test for pathogens infecting the upper respiratory tract. According to the Taiwan Centers for Disease Control guideline, we should retest the positive specimen in other genes. Hence, we suggest the specimens should be confirmed by other genes (for example, *E*, *Rdrp*, *N1*, *N3*, and *ORF1ab*). Furthermore, at US CDC, the *N2* gene was the confirmed target for the SARS-CoV-2 positive specimens.

Despite these limitations, there are several advantages of using our LDT multiplex RT-PCR assay to detect both SAR-CoV-2 and other upper respiratory pathogens. We used the LabTurbo AIO 48 system as a sample-to-result open platform, that is, from RNA extraction to nucleic acid amplification. The LabTurbo AIO system was combined with RNA extraction and RT-PCR thermocyclers. This could improve the robustness of extracted RNA to prepared master mix containing the desired primer and probe. Hence, the multiplex rRT-PCR we developed in this study could be applicable in other real-time PCR thermocyclers. We believe that this assay might be applies to other real-time PCR machines or sample-to-result platforms with open channels or open systems. Additionally, the multiplex PCR assay described here might serve as an alternative tool in clinical diagnostic laboratories for routine SARS-CoV-2 and influenza virus detection in the future when SARS-CoV-2 might gradually evolve into an endemic flu-like virus. Moreover, using the same reaction to detect SARS-CoV-2, influenza A/B virus, and RSV might help overcome the problem of shortage of supplies for nucleic acid extraction and PCR diagnostic reagents/equipment during the current COVID-19 pandemic.

METHODS

Specimen collection

Clinical upper respiratory samples were collected from May 1 to July 4, 2021. The retrospective specimens contained 102 SARS-CoV-2 positive and 550 negative samples, which were also confirmed using the WHO protocol described previously [24]. The *E* and *Rdrp* genes were confirmed in all positive samples by the central laboratory of the Taiwan Centers for Disease Control and Prevention, as reference data. This study was registered on March 20, 2021 and approved by the TSGH Institutional Review Board (approval number: C202005041). We tested all 652 nasopharyngeal swab specimens collected from patients suspected of having COVID-19, using LIBO Specimen Collection and Transport Swab Kits with Universal Transport Medium (New Taipei City, Taiwan). Influenza A and B- and RSV-positive specimens were confirmed using the BioFire® respiratory panel 2.1 (RP2.1) assay. The same specimens were detected by two clinical laboratories: TSGH (Taipei City, Taiwan) and CGH (Taipei City) using the same protocol and platform.

Assessment using the multiplex assay on the LabTurbo AIO 48 open system

In this study, we designed a multiplex PCR test that was performed on the LabTurbo AIO 48 system using specific primers and probes to simultaneously detect SARS-CoV-2, influenza A/B virus, and RSV in a well. The total viral nucleic acid was extracted from each swab in a universal viral transport medium (500 µL) to a final eluate volume of 60 µL using the LabTurbo Virus Mini Kit (Cat. No. LVN48-300) and an automated LabTurbo AIO open system. The Luna® Probe One-Step RT-qPCR Kit (No ROX, New England Biolabs), comprising reverse transcriptase and 2× PCR master mix, was used according to the RNA testing kit instructions. For analysis on the LabTurbo AIO open system, each 25-µL reaction mixture contained 12.5 µL of 2× PCR master mix, 4 µL of primer/probe mixture, 1.25 µL of reverse transcriptase, 1.25 µL of RNase-free water, and 6 µL of extracted RNA. SARS-CoV-2, influenza A/B virus, and RSV were detected using the

following thermal cycling conditions: 50°C for 10 min, 95°C for 2 min, and 45 cycles at 95°C for 10 s, 55°C for 25 s, and 64°C for 32 s. Here, we detected the *N2* gene in the SARS-CoV-2 genome, *M* gene in influenza A/B virus, *N* gene in RSV, and human ribonuclease *P* gene (RP) [25], which was also included as an internal control (Supplementary Table 2).

Assessment of analytical sensitivity

To validate the sensitivity of the designed multiplex assay, we tested all these pathogens (SARS-CoV-2, influenza A (subtypes H1, H1N1, and H3), influenza B, and RSV (subtypes A and B)) in one multiplex RT-PCR on the AIO48 open system. RNA controls (Vircell, Granada, Spain) of known concentrations were used to prepare several dilutions to determine the LoD by repeating the experiments 20 times. We defined limit of detection (LoD) as the minimum concentration with a positive detection rate of 95%. The analytical sensitivity of the LabTurbo AIO 48 tests was defined as the lowest dilution at which all replicates were identified as positive (C_t < 35) for SARS-CoV-2.

Evaluation of specificity

The specificity of multiplex master mix on the LabTurbo AIO platform was evaluated using viral cultures from the Taiwan CDC Viral Infection Contract Laboratory. We tested some common respiratory viruses (such as parainfluenza virus and enterovirus) to ensure that the master mix designed in this study could distinguish the virus of interest.

Comparison of the performance using clinical specimens

To validate the performance of the multiple PCR assay designed in this study to detect SARS-CoV-2 (*N2* gene), all specimens were subjected to rRT-PCR again for the *E* and *Rdrp* genes, per the WHO panel. The original samples were confirmed those genes as the reference genes in TSGH. The same specimens were detected by two medical centers: TSGH and CGH using the same protocol on the LabTurbo AIO48 open platform. The C_t value of <35 was defined as a positive result for the pathogen. Each sample had an internal control (*Rnase P* gene). The external control comprised RNA spike-in mix as the positive control and H_2O as the negative control.

Whole-genome sequencing of SARS-CoV-2

Ovation RNA-Seq System V2 (Nugen Technologies, San Carlos, CA, USA) was used to synthesize cDNA, which was then processed into a library as described previously [26]. WGS was performed as described previously [27]. Briefly, whole-genome sequences of the SARS-CoV-2 isolates (TSGH-42 and TSGH-43) were obtained following the protocol of the Illumina TruSeq Stranded mRNA Library Prep Kit to enrich SARS-CoV-2 cDNA using multiplex RT-PCR amplicons. Next-generation sequencing was performed on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA). Paired-end read assemblies of the whole virus genome sequence were formed using SPAdes assembler with SARS-CoV-2 isolate Wuhan-Hu-1, complete genome (NC_045512.2) to run the genome-guide assembly pipeline.

Phylogenetic relationship analysis

To identify pathogen evolution relationships, assembly sequences were uploaded to the Nextclade website (https://clades.nextstrain.org/) developed by Nextstrain [28]. TSGH sequences were aligned to the reference sequences of major clades of SARS-CoV (20I) grouped using Nextstrain and phylogenetic tree annotated with these alignment definitions, using lists of grouped clade-defining mutations via Augur workflow [29] supported by Nextclade. The results are shown in Supplementary Figure 1.

Classification of SARS-CoV-2 variant from positive specimen

To identify the variant type of SARS-CoV-2-positive specimens, we used the commercial kit developed by TIB Molbiol (Berlin, Germany). This kit can be used to rapidly detect SARS-CoV-2 VOC using extracted RNA with VirSNiP SARS-CoV-2 Spike N501Y and Spike del H69/V70. It was used to detect the mutations of SARS-CoV-2 using real-time RT-PCR post-melting curve analysis on LightCycler 480 (Roche Molecular Systems, Inc.) according to the manufacturer's instructions. The results were read following the manufacturer's instructions. The results were consistent with those of the rapid detection of SARS-CoV-2 variant.

AUTHOR CONTRIBUTIONS

Conceptualization: Jung-Chung Lin, Kuo-Ming Ye, Chien-Wen Chen, Ya-Sung Yang, and Feng-Yee Chang; Data curation: Hsing-Yi Chung and Ming-Jr Jian; Formal analysis: Hsing-Yi Chung and Ming-Jr Jian; Investigation: Hsing-Yi Chung, Chih-Kai Chang, En-Sung Chen, Mei-Hsiu Yang, Kuo-Sheng Hung, and Cherng-Lih Perng; Methodology: Chih-Kai Chang, Shan-Shan Hsieh, and Cherng-Lih Perng; Supervision: Hung-Sheng Shang, Jung-Chung Lin, Kuo-Ming Ye, Chien-Wen Chen, Feng-Yee Chang, Kuo-Sheng Hung, Sheng-Hui Tang, and Cherng-Lih Perng; Validation:

Hung-Sheng Shang; Writing – original draft: Hsing-Yi Chung and Ming-Jr Jian; Writing – review and editing: Hung-Sheng Shang.

CONFLICTS OF INTEREST

The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication. The authors declare no conflicts of interest.

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REFERENCES

1. Kirby T. New variant of SARS-CoV-2 in UK causes surge of COVID-19. Lancet Respir Med. 2021; 9:e20–21. https://doi.org/10.1016/S2213-2600(21)00005-9 PMID:33417829

2. Kidd M, Richter A, Best A, Cumley N, Mirza J, Percival B, Mayhew M, Megram O, Ashford F, White T, Moles-Garcia E, Crawford L, Bosworth A, et al. S-Variant SARS-CoV-2 Lineage B1.1.7 Is Associated With Significantly Higher Viral Load in Samples Tested by TaqPath Polymerase Chain Reaction. J Infect Dis. 2021; 223:1666–70. https://doi.org/10.1093/infdis/jiab082 PMID:33580259

3. Nordling TEM, Wu YH. Taiwan on track to end third COVID-19 community outbreak. medRxiv. 2021. [Preprint]. https://doi.org/10.1101/2021.06.20.21259178

4. Hsu CY, Wang JT, Huang KC, Fan AC, Yeh YP, Chen SL. Household transmission but without the community-acquired outbreak of COVID-19 in Taiwan. J Formos Med Assoc. 2021 (Suppl 1); 120:S38–45. https://doi.org/10.1016/j.jfma.2021.04.021 PMID:33994234

5. Perng CL, Jian MJ, Chang CK, Lin JC, Yeh KM, Chen CW, Chiu SK, Chung HY, Wang YH, Liao SJ, Li SY, Hsieh SS, Tsai SH, et al. Novel rapid identification of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by real-time RT-PCR using BD Max Open System in Taiwan. PeerJ. 2020; 8:e9318. https://doi.org/10.7717/peerj.9318 PMID:32596046

6. Avadhanula V, Nicholson EG, Ferlic-Stark L, Piedra FA, Blunck BN, Fragoso S, Bond NL, Santarcangelo PL, Ye X, McBride TJ, Aideyan LO, Patel KD, Maurer L, et al. Viral Load of Severe Acute Respiratory Syndrome Coronavirus 2 in Adults During the First and Second Wave of Coronavirus Disease 2019 Pandemic in Houston, Texas: The Potential of the Superspreader. J Infect Dis. 2021; 223:1528–37. https://doi.org/10.1093/infdis/jiab097 PMID:33585934

7. Mancini F, Barbanti F, Scaturro M, Fontana S, Di Martino A, Marsili G, Puzelli S, Calzoletti L, Facchini M, Di Mario G, Fabiani C, Bella A, Riccardo F, et al, and Istituto Superiore di Sanità (ISS) COVID-19 Team. Multiplex Real-Time Reverse-Transcription Polymerase Chain Reaction Assays for Diagnostic Testing of Severe Acute Respiratory Syndrome Coronavirus 2 and Seasonal Influenza Viruses: A Challenge of the Phase 3 Pandemic Setting. J Infect Dis. 2021; 223:765–74. https://doi.org/10.1093/infdis/jiaa658 PMID:33080031

8. Wollschläger P, Todt D, Gerlitz N, Pfaender S, Bollinger T, Sing A, Dangel A, Ackermann N, Korn K, Ensser A, Steinmann E, Buhl M, Steinmann J. SARS-CoV-2 N gene dropout and N gene Ct value shift as indicator for the presence of B.1.1.7 lineage in a commercial multiplex PCR assay. Clin Microbiol Infect. 2021; 27:1353.e1–1353.e5. https://doi.org/10.1016/j.cmi.2021.05.025 PMID:34044153

9. Wu X, Cai Y, Huang X, Yu X, Zhao L, Wang F, Li Q, Gu S, Xu T, Li Y, Lu B, Zhan Q. Co-infection with SARS-CoV-2 and Influenza A Virus in Patient with Pneumonia, China. Emerg Infect Dis. 2020; 26:1324–26. https://doi.org/10.3201/eid2606.200299 PMID:32160148

10. Chen Z, Li X, Li J, Zhang S, Zhou P, Yu X, Ren Y, Wang J, Zhang L, Li Y, Wu B, Hou Y, Zhang K, et al. A COVID-19 risk score combining chest CT radiomics and clinical characteristics to differentiate COVID-19 pneumonia from other viral pneumonias. Aging (Albany NY). 2021; 13:9186–224. https://doi.org/10.18632/aging.202735 PMID:33713401

11. Tan X, Zhang S, Xu J, Zhou M, Huang Q, Duan L, Lv Z, Xia H, Xiao W, Yin Z, Jin Y. Comparison of clinical characteristics among younger and elderly deceased patients with COVID-19: a retrospective study. Aging (Albany NY). 2020; 13:16–26. https://doi.org/10.18632/aging.202139 PMID:33323556

12. Wang W, He D, Chen J, Zhang Z, Wang S, Jiang Y, Wei J. Correction for: Circular RNA Plek promotes fibrogenic activation by regulating the miR-135b-

5p/TGF-βR1 axis after spinal cord injury. Aging (Albany NY). 2021; 13:16897. https://doi.org/10.18632/aging.203271 PMID:34192667

13. Leung EC, Chow VC, Lee MK, Tang KP, Li DK, Lai RW. Evaluation of the Xpert Xpress SARS-CoV-2/Flu/RSV Assay for Simultaneous Detection of SARS-CoV-2, Influenza A and B Viruses, and Respiratory Syncytial Virus in Nasopharyngeal Specimens. J Clin Microbiol. 2021; 59:e02965–20. https://doi.org/10.1128/JCM.02965-20 PMID:33436456

14. Chung HY, Jian MJ, Chang CK, Lin JC, Yeh KM, Chen CW, Chiu SK, Wang YH, Liao SJ, Li SY, Hsieh SS, Tsai SH, Perng CL, et al. Novel dual multiplex real-time RT-PCR assays for the rapid detection of SARS-CoV-2, influenza A/B, and respiratory syncytial virus using the BD MAX open system. Emerg Microbes Infect. 2021; 10:161–66. https://doi.org/10.1080/22221751.2021.1873073 PMID:33410371

15. Davies NG, Abbott S, Barnard RC, Jarvis CI, Kucharski AJ, Munday JD, Pearson CAB, Russell TW, Tully DC, Washburne AD, Wenseleers T, Gimma A, Waites W, et al, and CMMID COVID-19 Working Group, and COVID-19 Genomics UK (COG-UK) Consortium. Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England. Science. 2021; 372:eabg3055. https://doi.org/10.1126/science.abg3055 PMID:33658326

16. Shen L, Bard JD, Triche TJ, Judkins AR, Biegel JA, Gai X. Rapidly emerging SARS-CoV-2 B.1.1.7 sub-lineage in the United States of America with spike protein D178H and membrane protein V70L mutations. Emerg Microbes Infect. 2021; 10:1293–99. https://doi.org/10.1080/22221751.2021.1943540 PMID:34125658

17. Buchan SA, Tibebu S, Daneman N, Whelan M, Vanniyasingam T, Murti M, Brown KA. Increased household secondary attacks rates with Variant of Concern SARS-CoV-2 index cases. Clin Infect Dis. 2021. [Epub ahead of print]. https://doi.org/10.1093/cid/ciab496 PMID:34105720

18. Jassat W, Mudara C, Ozougwu L, Tempia S, Blumberg L, Davies MA, Pillay Y, Carter T, Morewane R, Wolmarans M, von Gottberg A, Bhiman JN, Walaza S, et al, and DATCOV Author Group. Increased mortality among individuals hospitalised with COVID-19 during the second wave in South Africa. medRxiv. 2021. [Preprint]. https://doi.org/10.1101/2021.03.09.21253184

19. Davies NG, Jarvis CI, Edmunds WJ, Jewell NP, Diaz-Ordaz K, Keogh RH, and CMMID COVID-19 Working Group. Increased mortality in community-tested cases of SARS-CoV-2 lineage B.1.1.7. Nature. 2021; 593:270–74. https://doi.org/10.1038/s41586-021-03426-1 PMID:33723411

20. Sockrider M, Jamil S, Santhosh L, Carlos WG. COVID-19 Infection versus Influenza (Flu) and Other Respiratory Illnesses. Am J Respir Crit Care Med. 2020; 202:P27–28. https://doi.org/10.1164/rccm.2020C16 PMID:33021812

21. Lansbury L, Lim B, Baskaran V, Lim WS. Co-infections in people with COVID-19: a systematic review and meta-analysis. J Infect. 2020; 81:266–75. https://doi.org/10.1016/j.jinf.2020.05.046 PMID:32473235

22. Abduljalil JM. Laboratory diagnosis of SARS-CoV-2: available approaches and limitations. New Microbes New Infect. 2020; 36:100713. https://doi.org/10.1016/j.nmni.2020.100713 PMID:32607246

23. Waggoner JJ, Stittleburg V, Pond R, Saklawi Y, Sahoo MK, Babiker A, Hussaini L, Kraft CS, Pinsky BA, Anderson EJ, Rouphael N. Triplex Real-Time RT-PCR for Severe Acute Respiratory Syndrome Coronavirus 2. Emerg Infect Dis. 2020; 26:1633–35. https://doi.org/10.3201/eid2607.201285 PMID:32294051

24. Jian MJ, Chung HY, Chang CK, Lin JC, Yeh KM, Chiu SK, Wang YH, Liao SJ, Li SY, Hsieh SS, Perng CL, Chang FY, Shang HS. Novel automated sample-to-result SARS-CoV-2 laboratory-developed RT-PCR assay for high-throughput testing using LabTurbo AIO 48 system. Clin Chim Acta. 2021; 514:54–58. https://doi.org/10.1016/j.cca.2020.12.003 PMID:33316217

25. Lu X, Wang L, Sakthivel SK, Whitaker B, Murray J, Kamili S, Lynch B, Malapati L, Burke SA, Harcourt J, Tamin A, Thornburg NJ, Villanueva JM, Lindstrom S. US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. Emerg Infect Dis. 2020; 26:1654–65. https://doi.org/10.3201/eid2608.201246 PMID:32396505

26. Gong YN, Tsao KC, Hsiao MJ, Huang CG, Huang PN, Huang PW, Lee KM, Liu YC, Yang SL, Kuo RL, Chen KF, Liu YC, Huang SY, et al. SARS-CoV-2 genomic surveillance in Taiwan revealed novel ORF8-deletion mutant and clade possibly associated with infections in Middle East. Emerg Microbes Infect. 2020; 9:1457–66. https://doi.org/10.1080/22221751.2020.1782271 PMID:32543353

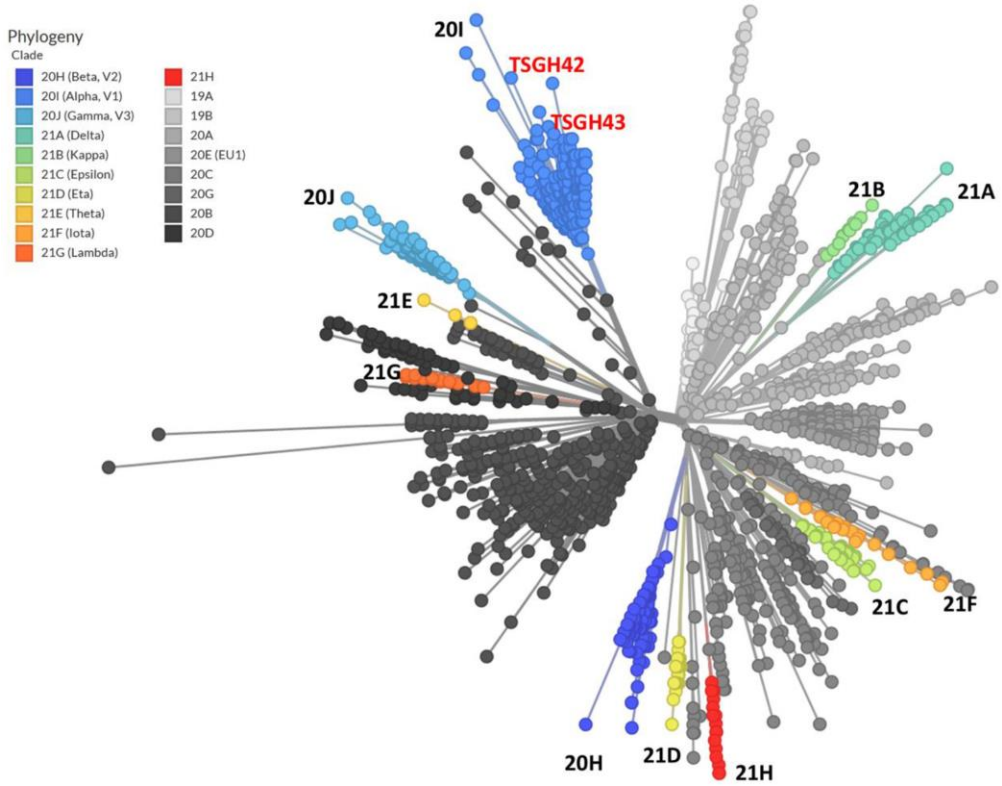
27. Jian MJ, Chung HY, Chang CK, Hsieh SS, Lin JC, Yeh KM, Chen CW, Chang FY, Chiu SK, Hung KS, Liu MT, Yang JR, Perng CL, Shang HS. Investigation of One Familial Cluster of COVID-19 in Taiwan: Differentiation of Genetic Variation Among Isolates and Implications for Epidemiological Investigation and Surveillance by Genomic Assay. Infect Drug Resist. 2021; 14:971–77. https://doi.org/10.2147/IDR.S298451 PMID:33737819

28. Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, Sagulenko P, Bedford T, Neher RA. Nextstrain: real-time tracking of pathogen evolution. Bioinformatics. 2018; 34:4121–23. https://doi.org/10.1093/bioinformatics/bty407 PMID:29790939

29. Huddleston J, Hadfield J, Sibley TR, Lee J, Fay K, Ilcisin M, Harkins E, Bedford T, Neher RA, Hodcroft EB. Augur: a bioinformatics toolkit for phylogenetic analyses of human pathogens. J Open Source Softw. 2021; 6:2906. https://doi.org/10.21105/joss.02906 PMID:34189396

SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. The clade of SARS-CoV-2 (TSGH42 and TSGH43) collected form retrospective positive specimens in this study. The clade belongs to SARS-CoV-2 B.1.1.7 variant (20I).

Supplementary Tables

Supplementary Table 1. Assessment of Limit of detection for mixed pathogens of respiratory tract virus in multiplex RT-PCR.

Mixed pathogen (RNA copies per PCR)	SARS-CoV-2 RNA copies per PCR (No. of replicates detected at each dilution/total no. of replicates at indicated no. of copies per PCR (percentage))						
	300	75	24	18.8	9.4	3.8	1.9
Influenza A H1 (300)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	11/20 (55)	0/20 (0)
Influenza A H1 (24)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	12/20 (60)	0/20 (0)
Influenza A H3 (300)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	12/20 (60)	0/20 (0)
Influenza A H3 (24)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	10/20 (50)	0/20 (0)
Influenza A H1N1(300)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	12/20 (60)	0/20 (0)
Influenza A H1N1(24)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	11/20 (55)	0/20 (0)
Influenza B (300)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	10/20 (50)	0/20 (0)
Influenza B (24)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	12/20 (60)	0/20 (0)
RSV subtype A (300)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	11/20 (55)	0/20 (0)
RSV subtype A (24)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	12/20 (60)	0/20 (0)
RSV subtype B (300)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	11/20 (55)	0/20 (0)
RSV subtype B (24)	20/20 (100)	20/20 (100)	20/20 (100)	20/20 (100)	19/20 (95)	12/20 (60)	0/20 (0)

Supplementary Table 2. Primers and probe used in this study.

Primer name	Description	Primer sequence (5′→3′)	References
2019-nCov_N2-F	2019-nCoV-forward sequence	TTACAAACATTGGCCGCAAA	[25]
2019-nCov_N2-R	2019-nCoV reverse sequence	GCGCGACATTCCGAAGAA	[25]
2019-nCov_N2-P	2019-nCoV probe	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ-1	[25]
InfA-F	Influenza A virus forward sequence	CCMAGGTCGAAACGTAYGTTCTCTCTATC	[14]
InfA-R	Influenza A virus reverse sequence	TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA	[14]
InfA-P	Influenza A virus probe	VIC-ATYTCGGCTTTGAGGGGGCCTG-BBQ	[14]
InfB-F	Influenza B virus forward sequence	GAGACACAATTGCCTACTTGCTT	[14]
InfB-R	Influenza B virus reverse sequence	TTCTTTCCCACCAAACCAAC	[14]
InfB-P	Influenza B virus probe	Cy5-AGAAGATGGAGAAGGCAAAGCAGAACTAGC-BBQ	[14]
RSV-N-F	Respiratory syncytial virus forward sequence	CTGTCATCCAGCAAATACAC	[14]
RSV-N-R	Respiratory syncytial virus reverse sequence	GCATATAACATACCTATTAAYCC	[14]
RSV-N-P	Respiratory syncytial virus probe	Texas red—ACAGGAGATARTATTGAYACTCCYAAT-BBQ	[14]
RP-F	RNase P forward sequence	AGA TTT GGA CCT GCG AGC G	[25]
RP-R	RNase P reverse sequence	GAG CGG CTG TCT CCA CAA GT	[25]
RP-P	RNase P probe	Cy5.5-TTC TGA CCT GAA GGC TCT GCG CG-BBQ	[25]

Research Paper

Decreased TMPRSS2 expression by SARS-CoV-2 predicts the poor prognosis of lung cancer patients through metabolic pathways and immune infiltration

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread around the world and became a global pandemic in 2020. One promising drug target for SARS-CoV-2 is the transmembrane protease serine 2 (TMPRSS2). This study was designed to explore the expression status, prognostic significance and molecular functions of TMPRSS2 in lung cancer. TMPRSS2 expression was investigated using the TIMER, Oncomine, UALCAN, GEO, HPA and TCGA databases. The prognostic value of TMPRSS2 was examined using Cox regression and a nomogram. KEGG, GO and GSEA were performed to investigate the cellular function of TMPRSS2 in lung cancer. The relationship between TMPRSS2 and immune infiltration was determined using the TIMER and CIBERSORT algorithms. TMPRSS2 mRNA and protein expression was significantly reduced in lung cancer. Decreased TMPRSS2 expression and increased DNA methylation of TMPRSS2 were associated with various clinicopathological parameters in patients with lung cancer. Low TMPRSS2 mRNA expression also correlated with poor outcome in lung cancer patients. Moreover, a nomogram was constructed and exhibited good predictive power for the overall survival of lung cancer patients. KEGG and GO analyses and GSEA implied that multiple immune- and metabolism-related pathways were significantly linked with TMPRSS2 expression. Intriguingly, TMPRSS2 expression associated with immune cell infiltration in lung cancer. More importantly, TMPRSS2 expression was markedly decreased in SARS-CoV-infected cells. These findings indicate that TMPRSS2 may be a promising prognostic biomarker and therapeutic target for lung cancer through metabolic pathways and immune cell infiltration.

INTRODUCTION

COVID-19 (coronavirus disease 2019) has emerged from infection with SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) and has created a global epidemic with over 257 million patients in most countries of the world and more than 5.1 million deaths (updated on 24 November 2021) [1–3]. Recently studies have demonstrated that both transmembrane protease serine 2 (TMPRSS2) and angiotensin I converting enzyme 2 (ACE2) are crucial for the entry of SARS-CoV-2 into host cells [4–6]. Both TMPRSS2 and ACE2

are expressed in lung tissues, as implicated in the clinical manifestations of COVID-19 [4–6]. TMPRSS2 is also expressed in other tissues, such as the prostate epithelium, cardiac endothelium, digestive tract and kidney, indicating that these organs may be the most susceptible targets for SARS-CoV-2 infection [7, 8]. Consistent with these observations, SARS-CoV-2 infection can result in multisystemic, life-threatening complications. Similar to SARS and MERS-CoV, SARS-CoV-2 mainly affects the function of the lower respiratory tract [9, 10]. In more severe cases, it can induce acute respiratory distress syndrome and severe lung damage, leading to inflammation and pulmonary vasculopathy [2, 9].

Lung cancer is the most frequent malignancy and the leading cause of cancer-related death worldwide [10]. NSCLC (Non-small cell lung cancer) is the most common pathological type of lung cancer and is responsible for 85% of all lung cancers [10, 11]. Radiotherapy, chemotherapy, surgical resection, and immunotherapy are common therapies employed to treat lung cancer [10, 11]. Due to problems in early diagnosis, patients with NSCLC are often diagnosed at advanced stages [10, 11]. Patients with lung cancer are more vulnerable to various infections due to poor healthy condition, accompanying chronic diseases, and immunosuppression induced by tumor and/or antitumor therapies. Therefore, cancer patients who are infected by SARS-CoV-2 may suffer worse outcomes than other individuals [12]. Indeed, previous studies have demonstrated that cancer patients with coronavirus infections may be more susceptible to higher morbidity and mortality rates. In a study at a hospital in Wuhan, China, cancer patients accounted for 1% of the total prevalence of COVID-19, which is substantially higher than the 0.29% of the total incidence of cancer in the Chinese population [13, 14]. Lung cancer patients seem to be more susceptible to SARS-CoV-2 infection [12, 15]. Therefore, the association between immune infiltration in cancer patients and the susceptibility or severity of COVID-19 needs to be fully elucidated.

Previous studies have revealed that multiple viruses, such as influenza virus, Ebola virus, MERS-CoV, and SARS-CoV, use host cell proteases to facilitate the activation of their envelope glycoproteins [16]. The cleavage and activation of the spike protein (S protein) of SARS-CoV are regulated by TMPRSS2 [5, 6]. TMPRSS2 is a protease that belongs to the type II transmembrane serine protease family and is required to activate S protein to cause virus-cell membrane fusion and promote coronaviruses to inter into the host cell [5, 6]. Several animal models have demonstrated that TMPRSS2-KO (TMPRSS2-knockout) mice can be protected from severe pathology and death after influenza virus infection [16–19]. Knockout of TMPRSS2 prevents the spread of MERS-CoV and SARS-CoV in the airway of a mouse model by alleviating inflammatory cytokine production [17, 18]. The reduced TMPRSS2 expression alters the primary site of infection and the transmission of the virus in the airway, leading to less severe immunopathology [17–19]. In contrast, overexpression of certain TMPRSS2 variants in animals results in an increased risk of severe outcomes after infection with A (H1N1) pmd09 influenza [20].

Given that TMPRSS2 is a promising drug target for SARS-CoV-2, this study aimed to investigate the expression profile, determine the prognostic potential of TMPRSS2, and estimate the association between TMPRSS2 and immune cell infiltration in lung cancer. We observed that TMPRSS2 expression was decreased in lung cancer tissues compared with adjacent nontumor tissues. TMPRSS2 expression was reduced in different tumor stages and linked with lymph node metastasis. Subsequently, TMPRSS2 expression was negatively and significantly related with the prognosis of lung cancer patients. Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene Ontology (GO) analyses and gene set enrichment analysis (GSEA) demonstrated that various metabolic and immune-related pathways were strongly associated with TMPRSS2 expression. Moreover, there was a significant correlation between TMPRSS2 expression and the infiltration abundances of CD8+ T cells, CD4+ T cells, B cells, neutrophils, macrophages, and dendritic cells in lung cancer. Importantly, TMPRSS2 expression was significantly decreased during SARS-CoV-2 infection. These findings emphasize a notable role of TMPRSS2 in carcinogenesis.

RESULTS

TMPRSS2 expression across cancers

We first estimated TMPRSS2 expression at the gene transcription level in various human tissues and organs using the GTEx database. Consistent with previous studies, we observed that TMPRSS2 was highly expressed in internal tissues (small intestine, kidney, colon, lung, liver, esophagus, stomach and bladder), secretory tissues (pancreas, thyroid, breast, salivary gland, pituitary and skin) and reproductive tissues (prostate and testis) (Supplementary Figure 1A). We then investigated TMPRSS2 expression in common tumors and their adjacent normal tissues through the TIMER database. The TMPRSS2 mRNA levels in BRCA, COAD, KIRC, KIRP, HNSC, LUAD, LUSC, LIHC, READ and THCA were significantly reduced compared with those in corresponding adjacent normal tissues (Figure 1A). In contrast, a significant

increase in TMPRSS2 expression was observed in KICH, PRAD, and UCEC (Figure 1A). Moreover, TMPRSS2 mRNA expression levels in different cancer types were assessed through the Oncomine database. In 41 of the 43 unique analyses, TMPRSS2 expression was downregulated (Figure 1B). TMPRSS2 was significantly decreased in LUSC, LUAD, lung carcinoid tumor, small cell lung carcinoma, and large cell lung carcinoma (Figure 1C; Supplementary Figure 1B). Consistently, lower TMPRSS2 mRNA expression was

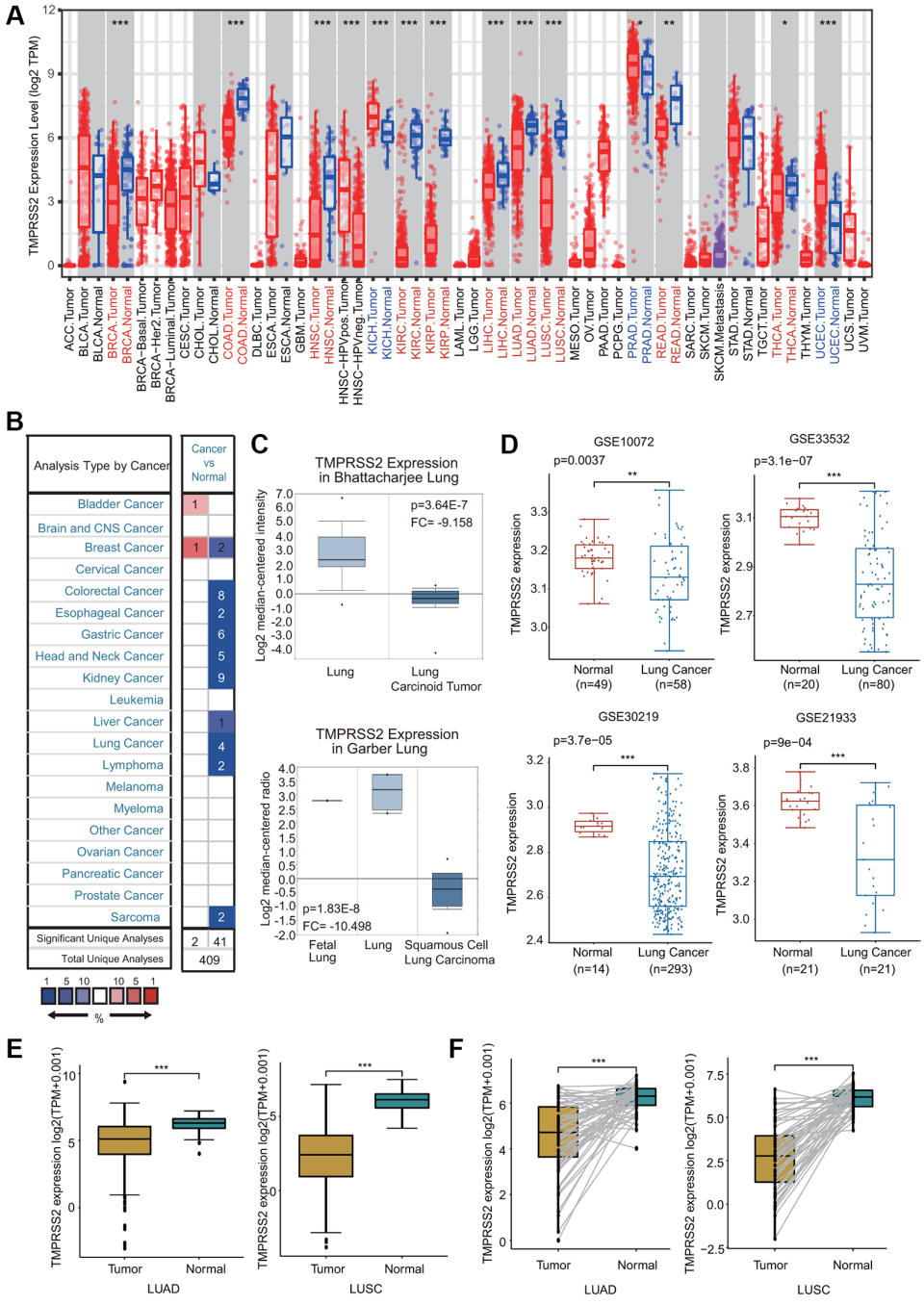

Figure 1. TMPRSS2 expression in lung cancer. (**A**) The mRNA expression of TMPRSS2 in different cancers from the TIMER database. (**B**) Upregulated or downregulated expression of TMPRSS2 in various tumors compared with normal tissues in the Oncomine database. (**C**) Box plots showing TMPRSS2 mRNA level in different types of lung cancer patients and normal individuals from the Oncomine database. (**D**) TMPRSS2 mRNA level in lung cancer patients and normal individuals in the GSE10072 (normal, *n* = 49; lung cancer, *n* = 58), GSE33532 (normal, *n* = 20; lung cancer, *n* = 80), GSE30219 (normal, *n* = 14; lung cancer, *n* = 293) and GSE21933 (normal, *n* = 21; lung cancer, *n* = 81) datasets. (**E**) TMPRSS2 expression is decreased in lung cancer (*n* = 81) compared with noncancerous adjacent tissues (*n* = 81) from the TCGA database. (**F**) TMPRSS2 expression in 58 and 50 matched LUAD and LUSC samples and adjacent normal lung tissues in the TCGA database was determined. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

found in four GEO cohorts, GSE10072, GSE33532, GSE30219 and GSE21933 (Figure 1D). Moreover, we compared TMPRSS2 expression in lung cancer using the TCGA dataset, and the results demonstrated that TMPRSS2 expression was significantly downregulated in lung cancer tissues (Figure 1E). TMPRSS2 expression in 58 and 50 paired LUAD and LUSC samples and corresponding adjacent normal samples was analyzed, and our results suggested a marked decrease in TMPRSS2 in lung cancer (Figure 1F). Additionally, we assessed TMPRSS2 expression in multiple cancer cell lines based on the CCLE database and found that TMPRSS2 expression was high in COAD, BRCA, PAAD, STAD and PRAD cells but low in AML, MESO, ALL and LUSC cells (Supplementary Figure 1C).

Correlation between TMPRSS2 expression and clinicopathological characteristics

We then examined the expression profiles of TMPRSS2 in lung cancer based on clinicopathological characteristics. Analysis mining of the UALCAN database revealed that TMPRSS2 expression was reduced in both female and male lung cancer patients (Figure 2A). According to cancer stage, significant downregulation of TMPRSS2 expression was observed in stage 1, 2, 3 and 4 LUAD and LUSC patients (Figure 2B). In terms of nodal metastasis status, TMPRSS2 expression was also greatly decreased in N0, N1, N2 and N3 in both LUSC and LUAD (Figure 2C). TMPRSS2 expression was lower in tumors from patients in different age groups (21–40, 41–60, 61–80 and 81–100 years) than in normal lung tissues (Supplementary Figure 2A). Moreover, TMPRSS2 expression in Asian, African-American and Caucasian was significantly decreased in LUSC patients (Supplementary Figure 2B). TMPRSS2 expression was dramatically downregulated in Caucasian and African-American LUAD patients (Supplementary Figure 2B). TMPRSS2 expression was similarly reduced in both TP53-mutant and TP53-nonmutant LUAD and LUSC patients (Supplementary Figure 2C). In summary, these results demonstrated that TMPRSS2 expression is significantly correlated with clinicopathological parameters in lung cancer patients.

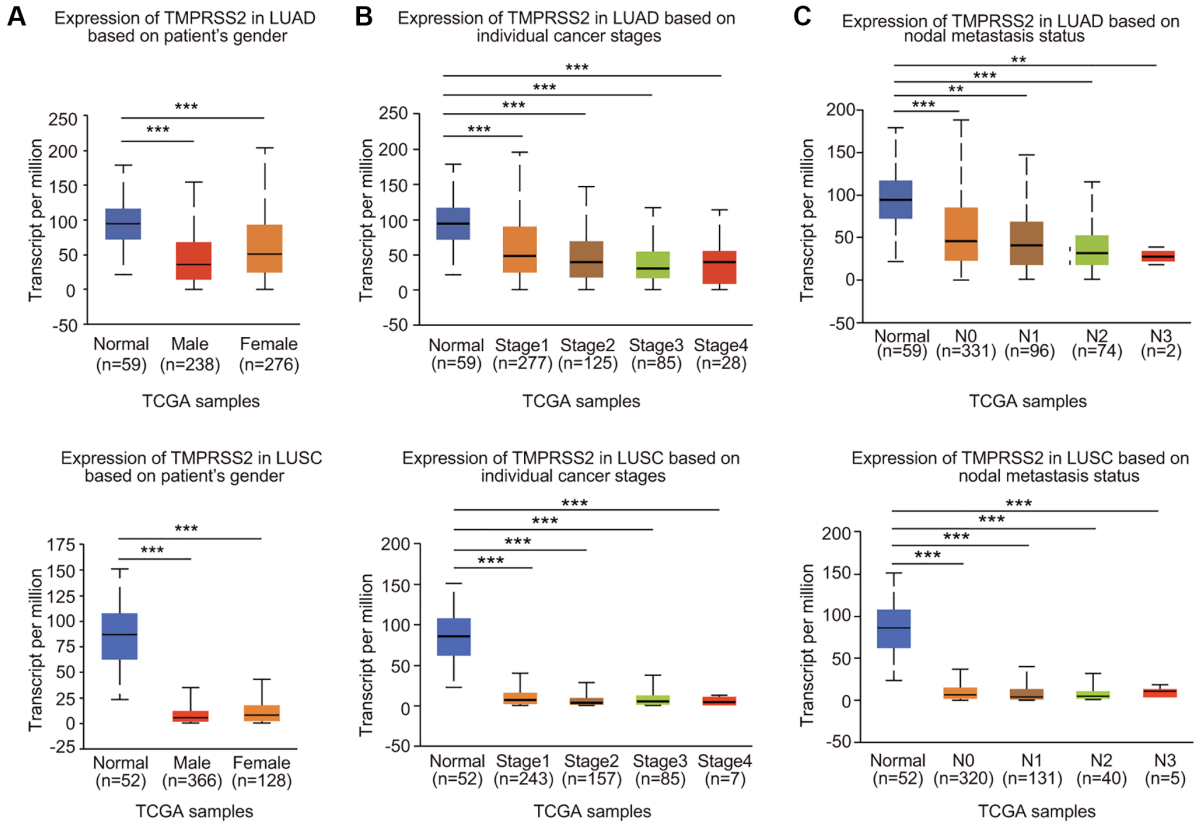

Figure 2. Relationship between TMPRSS2 expression and clinicopathological parameters of lung cancer patients. TMPRSS2 expression was assessed in (**A**) male and female LUAD (normal, *n* = 59; male, *n* = 238; female, *n* = 276) and LUSC (normal, *n* = 52; male, *n* = 366; female, *n* = 128) patients, (**B**) patients with different stages of LUAD (normal, *n* = 59; stage 1, *n* = 277; stage 2, *n* = 125, stage 3, *n* = 85; stage 4, *n* = 28) and LUSC (normal, *n* = 52; stage 1, *n* = 243; stage 2, *n* = 157, stage 3, *n* = 85; stage 4, *n* = 7), (**C**) patients with different nodal metastasis statuses of LUAD (normal, *n* = 59; N0, *n* = 331; N1, *n* = 96, N2, *n* = 74; N3, *n* = 2) and LUSC (normal, *n* = 52; N0, *n* = 320; N1, *n* = 131, N2, *n* = 40; N3, *n* = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TMPRSS2 is an independent predictor of prognosis in lung cancer

The impact of TMPRSS2 on the survival of lung cancer patients was analyzed with the PrognoScan and Kaplan–Meier plotter databases. Lung cancer patients with lower TMPRSS2 expression exhibited poor overall survival (OS), postprogression survival (PPS) and first-progression survival (FPS) according to the Kaplan–Meier plotter database (Figure 3A). In addition, the analysis results from the PrognoScan database indicated that decreased TMPRSS2 expression was linked with inferior OS and relapse-free survival (RFS) in different lung cancer cohort samples (Figure 3B). Thus, a low transcriptional level of TMPRSS2 was associated with an unfavorable prognosis.

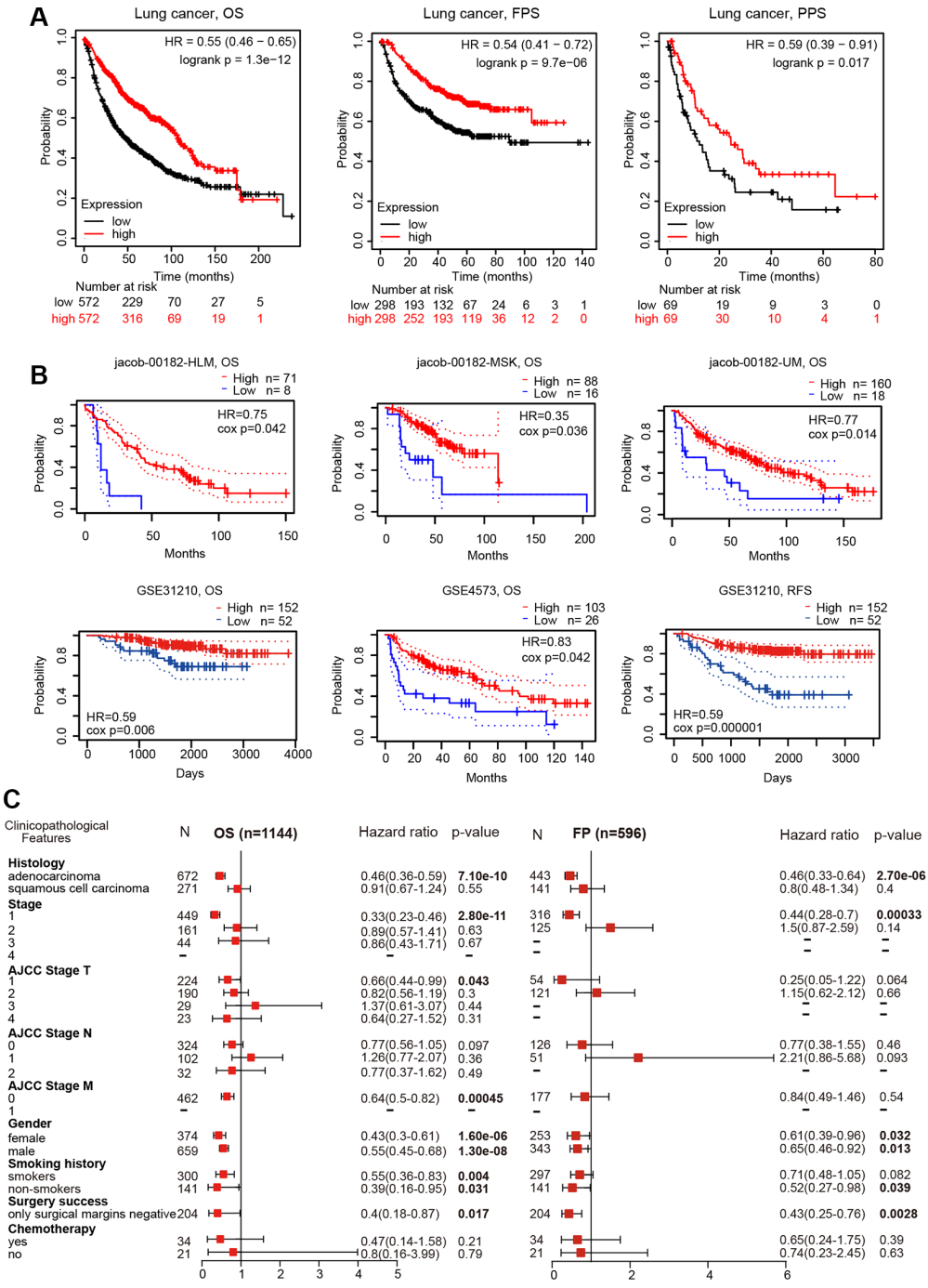

Figure 3. Prognostic value of TMPRSS2 expression in lung cancer. (**A**) The OS, FPS and PPS of lung cancer patients were obtained from the Kaplan–Meier plotter database. (**B**) The OS and RFS of lung cancer cohorts obtained through the Prognoscan database. (**C**) Forest plots showing the associations between TMPRSS2 expression and various clinicopathological features of patients with lung cancer.

Prognostic potential of TMPRSS2 according to different clinicopathological characteristics

To further explore the prognostic potential of TMPRSS2 expression in lung cancer, the relationships between TMPRSS2 expression and the clinical features of lung cancer patients were examined. Intriguingly, reduced TMPRSS2 expression was strongly related with worse OS and FPS in LUAD, but not in LUSC (Figure 3C), and decreased TMPRSS2 expression was obviously linked with OS and FPS in stage 1 lung cancer (Figure 3C). Additionally, there were significant associations between TMPRSS2 expression and poor OS in AJCC stage T-1 and stage M-0 lung cancer patients (Figure 3C). In the analysis by smoking history, downregulation of TMPRSS2 expression contributed to poor OS in both smokers and nonsmokers and poor FPS in nonsmokers (Figure 3C). With respect to sex, low TMPRSS2 expression was strongly linked with worse OS and FPS in female and male lung cancer patients (Figure 3C).

Conduction of univariate and multivariate Cox hazard regression analysis

We conducted univariate Cox and multivariate Cox regression analyses to explore whether TMPRSS2 expression was an independent prognostic factor that correlated with the OS of lung cancer patients. The results of univariate Cox regression analysis indicated that TMPRSS2 expression, age, T stage, N stage, M stage and radiation therapy were obviously correlated with the OS of lung cancer patients (Figure 4A). Moreover, the results of multivariate Cox regression analysis indicated that M stage and radiation therapy showed obvious correlations with the OS of lung cancer patients (Figure 4B). According to these results, TMPRSS2 can serve as an independent prognostic biomarker of OS when adjusted by other related variables.

Construction of a nomogram model

We then developed a novel nomogram model to predict the 1-, 3-, and 5-year OS rates of lung cancer patients

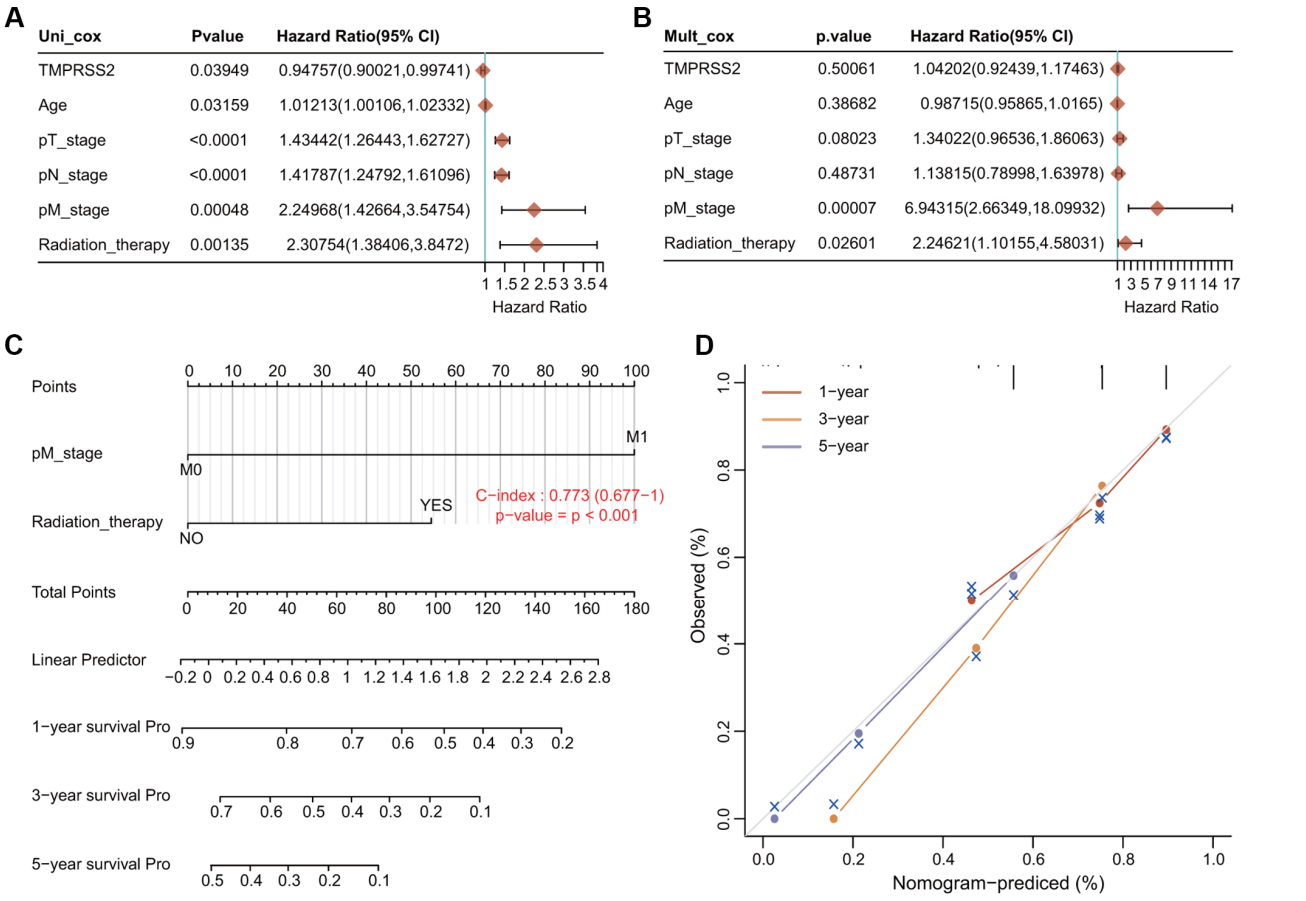

Figure 4. Establishment and validation of the prognostic nomogram. (**A**, **B**) Univariate and multivariate Cox regression analysis of clinicopathologic variables and TMPRSS2 in lung cancer. (**C**) Nomogram for predicting the 1-, 3-, and 5-year OS of lung cancer patients. (**D**) Calibration curves of 1-, 3-, and 5-year OS of lung cancer patients.

(Figure 4C). The C index (concordance index) of the prognostic nomogram is 0.773 (Figure 4C). The calibration plots for predicting the 1-, 3-, and 5-year OS rates of lung cancer patients also showed good agreement between the predicted and actual survival outcomes (Figure 4D).

The DNA methylation level and genetic alterations in TMPRSS2 in lung cancer

DNA methylation is known to be associated with gene expression and cancer development. We assessed the DNA methylation of TMPRSS2. Both LUAD and LUSC samples showed elevated levels of DNA methylation of TMPRSS2 (Figure 5A; Supplementary Figure 3A). Moreover, the DNA methylation level of TMPRSS2 was also greatly upregulated in LUAD and LUSC patients with different sexes, tumor stages, nodal metastasis statuses, ages and races (Figure 5A; Supplementary Figure 3A). According to the SurvivalMeth database, we also observed increased methylation levels in different CpG sites in the DNA of the TMPRSS2 gene (Figure 5B, 5C; Supplementary Figure 3B, 3C). The heatmap of the DNA methylation results for TMPRSS2 in LUAD and LUSC is shown in Figure 5D and Supplementary Figure 3D. Higher methylation level in CpG sites of the TMPRSS2 gene was linked with worse prognosis in LUAD and LUSC (Figure 5E; Supplementary Figure 3E).

cBioPortal was used to analyze the genetic alterations in TMPRSS2 in lung cancer. Genetic alterations in TMPRSS2 occurred in 1.2% of lung cancer patients (Supplementary Figure 4A). In LUAD, TMPRSS2 was mainly altered by mutation and amplification, whereas TMPRSS2 was mainly altered by deep deletion in LUSC and NSCLC (Supplementary Figure 4B). However, the Kaplan–Meier plotter results indicated that although the survival rate of patients without TMPRSS2 alterations appeared to be worse, there were no significant differences in OS, DFS, PFS or disease-specific survival (DSS) between patients with lung cancer with alterations in TMPRSS2 and those without alterations in TMPRSS2 (Supplementary Figure 4C).

Key candidate genes and proteins identified from the TMPRSS2 interactive network

A gene–gene interaction network for TMPRSS2 was constructed using the GeneMANIA database. The top three genes significantly correlated with TMPRSS2 were KDM3A, POU2F1 and SLC37A1 (Supplementary Figure 5A). To further estimate the functions of TMPRSS2, a protein–protein interaction (PPI) network was carried out through the STRING database. A total of 10 TMPRSS2-interacting proteins were identified (Supplementary Figure 5B). Among the 11 nodes, the three nodes with the highest degree centrality are AR, ACE2, and TMPRSS4 (Supplementary Figure 5B). Interestingly, two common hub genes were shown from the STRING and GeneMANIA databases: AR and SLC45A3. We then assessed the correlations between TMPRSS2 and these two proteins in the TIMER and GEPIA2 databases. TMPRSS2 expression was correlated with AR and SLC45A3 in LUAD and only correlated with AR in LUSC (Supplementary Figure 5C, 5D). We then examined the relationship between TMPRSS2 and other targets for COVID-19 therapy, including ACE2, AXL, CTSL and FURIN. TMPRSS2 was positively correlated with ACE2 and AXL in LUAD and LUSC but negatively associated with CTSL in LUAD (Supplementary Figure 5E).

KEGG and GO analyses of TMPRSS2

The functions of TMPRSS2 and the genes significantly associated with TMPRSS2 alterations were predicted by GO and KEGG analyses. A total of 300 coexpressed TMPRSS2 genes were used, and the top fifty genes that were positively and negatively associated with TMPRSS2 in LUSC and LUAD are shown (Figure 6A, 6B and Supplementary Figure 6A, 6B). Furthermore, the top 20 significant terms of GO enrichment analysis are presented. Regarding the biological process (BP) terms, the results showed that urogenital and renal system development and various metabolic processes were associated with TMPRSS2 in LUAD; multiple immune-related pathways, including humoral immune response, acute inflammatory response, positive regulation of cytokine secretion, and regulation of humoral immune response, were significantly correlated with TMPRSS2 in LUSC (Figure 6C, 6D). Regarding the molecular function (MF) terms, the results suggested that enzyme inhibitor activity, coenzyme binding and inorganic anion transmembrane transporter activity were associated with TMPRSS2 in LUAD; anion transmembrane transporter activity, carbohydrate binding and gated channel activity were associated with TMPRSS2 in LUSC (Supplementary Figure 6C, 6D). Regarding the cellular component (CC) terms, the results suggested that the apical part of the cell, collagen-containing extracellular matrix, and apical plasma membrane were related with TMPRSS2 in both LUAD and LUSC (Supplementary Figure 6E, 6F). Additionally, KEGG analysis results suggested that TMPRSS2 was involved in adrenergic signaling in cardiomyocytes, ECM-receptor interaction, hypertrophic cardiomyopathy (HCM), and bile secretion in lung cancer (Figure 6E, 6F).

GSEA revealed TMPRSS2-associated signaling pathways

GSEA was conducted to examine the TMPRSS2-associated signaling pathways that were differentially activated in lung cancer. The outcome implied that regarding the GO terms in LUSC, the top twenty signaling pathways affected by TMPRSS2 were mainly enriched in immune response-associated activities, including myeloid leukocyte activation, leukocyte

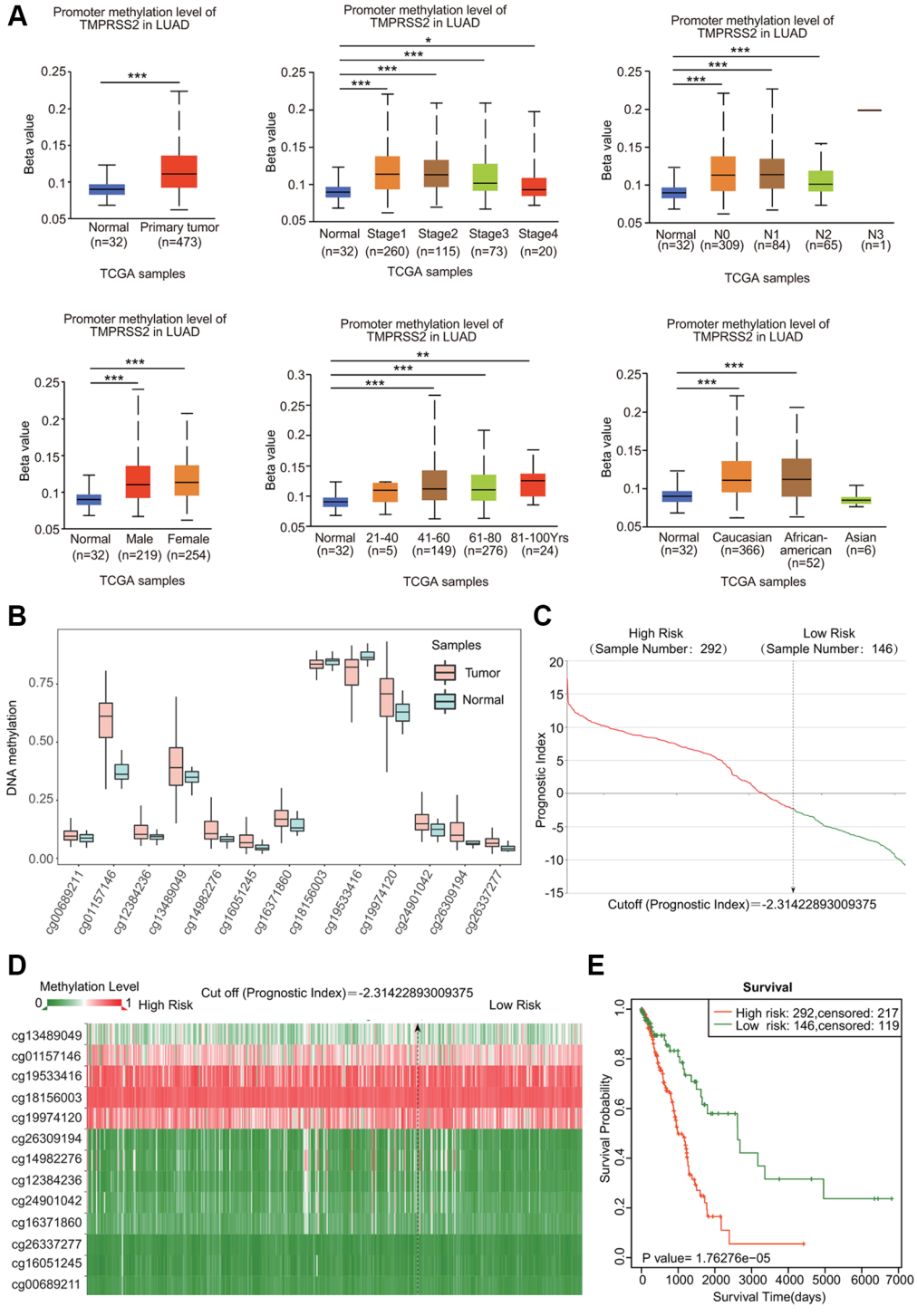

Figure 5. DNA methylation of TMPRSS2 in LUAD. (**A**) Associations of DNA methylation of TMPRSS2 with clinicopathological parameters of LUAD. (**B**) Methylation levels of TMPRSS2 in LUAD according to the SurvivalMeth database. (**C**) The distribution of prognostic index in LUAD. (**D**) Heatmap of DNA methylation of TMPRSS2 in LUAD. (**E**) The prognostic potential of DNA methylation of TMPRSS2 in LUAD based on the SurvivalMeth database. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

activation involved in the immune response, cell activation involved in the immune response, activation of the immune response, leukocyte mediated-immunity, immune effector process, cytokine production, immune response-activating cell surface receptor signaling pathway, and activation of the innate immune response (Figure 7A, 7B). Similarly, regarding the KEGG terms, the GSEA results indicated various immune functional gene sets that were enriched in both LUAD and LUSC, including Th17 cell differentiation, cytokine–cytokine receptor interaction, herpes simplex virus 1 infection, and the TNF signaling pathway (Figure 7C, 7D). These findings demonstrate that TMPRSS2 plays a critical role in the TME (tumor microenvironment).

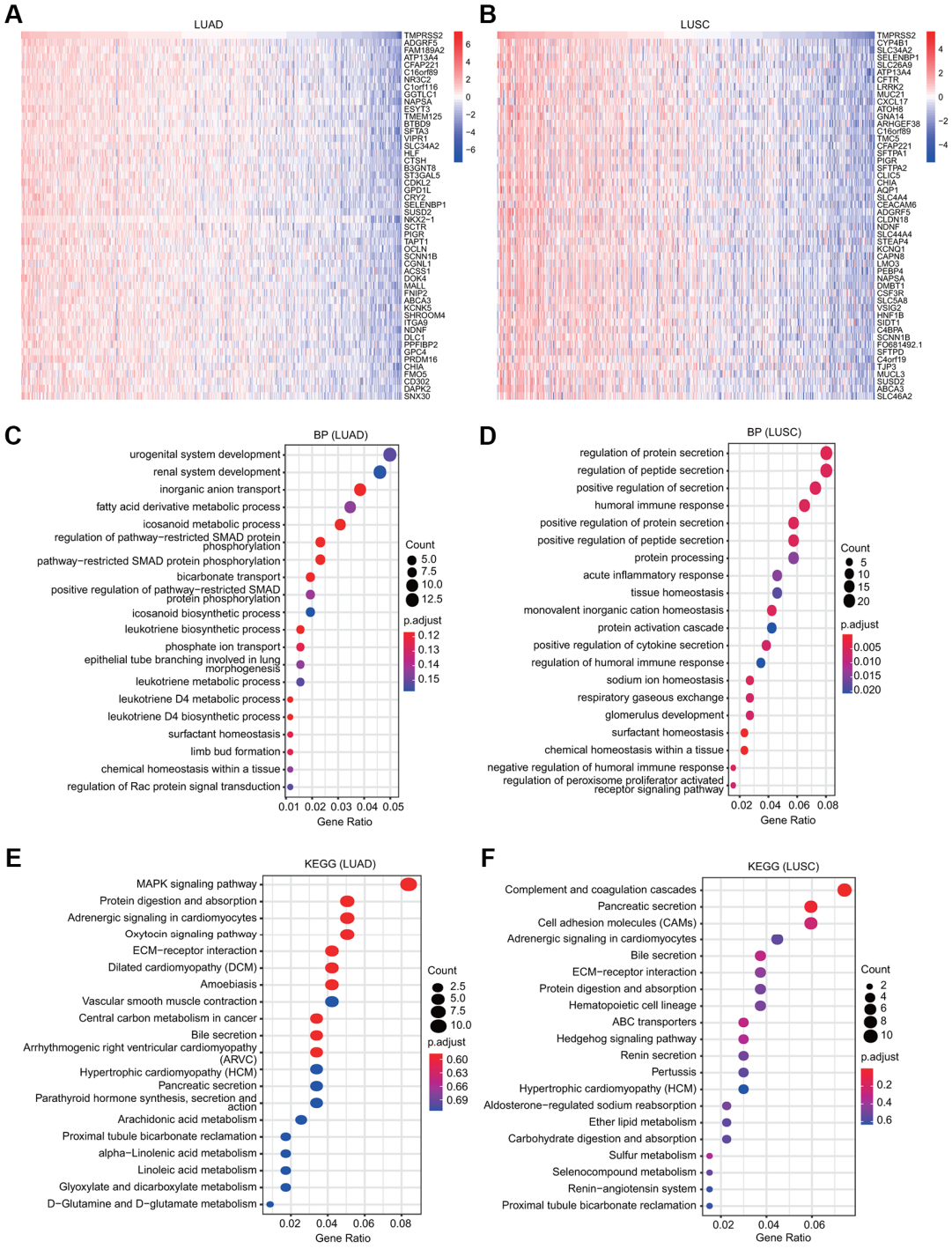

Figure 6. GO and KEGG analyses for TMPRSS2 in lung cancer. (**A**, **B**) Heatmaps showing the top 50 genes that were positively correlated with TMPRSS2 in LUAD and LUSC. (**C**, **D**) Top 20 enrichment terms in the BP category in LUAD and LUSC. (**E**, **F**) Top 20 pathways enriched in the KEGG analysis in LUAD and LUSC.

We further identified the associations between TMPRSS2 and human diseases to assist drug discovery using the Open Targets platform. We found that TMPRSS2 was associated with various human diseases, such as cardiovascular disease, endocrine system diseases, immune system disease, respiratory or thoracic disease, infectious diseases (COVID-19 and severe acute respiratory syndrome) (Supplementary Figure 7A) and cancer or benign tumors (prostate carcinoma, gastric adenocarcinoma, colon adenocarcinoma, rectal adenocarcinoma, small cell lung carcinoma, etc) (Supplementary Figure 7B).

The correlations between TMPRSS2 expression and immune cell infiltration in lung cancer

The potential immunological correlations of TMPRSS2 and immune cell infiltration were investigated. TMPRSS2 expression was significantly correlated with the infiltration levels of B cells, CD4+ T cells and neutrophils in LUAD (Figure 8A). TMPRSS2 expression was positively and significantly linked with the infiltrating levels of all six types of immune cells in LUSC (Figure 8A). According to TMPRSS2 expression, lung cancer patients were divided into low- and high-expression groups. The percentage abundance of tumor infiltrating immune cells in each sample is indicated using multiple colors for various types of immune cells using TIMER (Figure 8B). We observed that the infiltrating levels of B cells and CD4+ T cells were enhanced in the TMPRSS2 high-expression group compared with the low-expression group in LUAD (Figure 8C). Moreover, the infiltrating levels of CD4+ T cells, CD8+ T cells, B cells, neutrophils, macrophages, and dendritic cells were increased in the TMPRSS2 high-expression group compared with

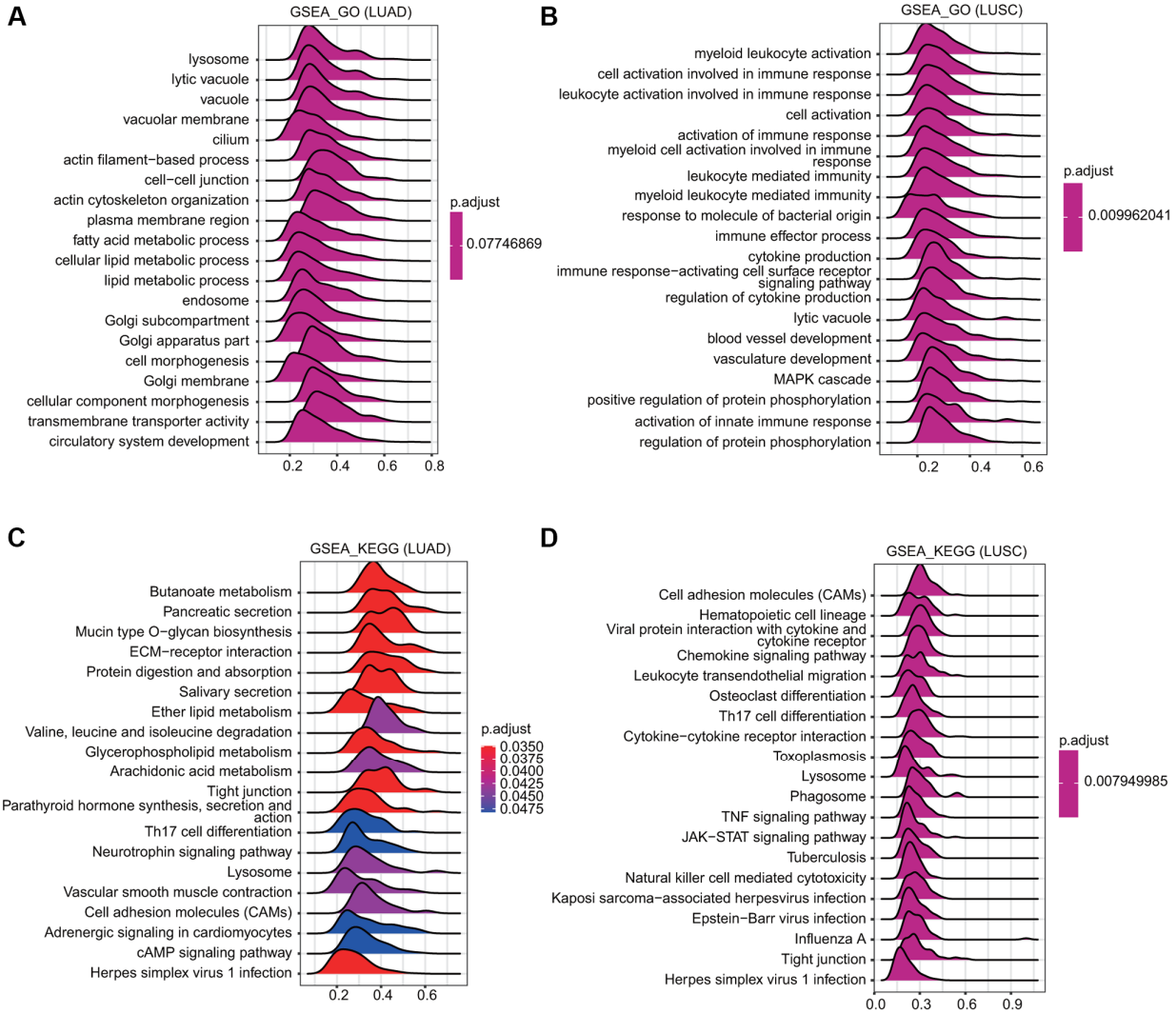

Figure 7. Merged enrichment plots obtained by GSEA. (**A**, **B**) Merged plots indicating the signaling pathways associated with TMPRSS2 expression according to GO analyses in LUAD and LUSC. (**C**, **D**) Merged plots indicating the signaling pathways associated with TMPRSS2 expression according to KEGG analyses in LUAD and LUSC.

the low-expression group in LUSC (Figure 8C). The correlations between TMPRSS2 expression and immune cell infiltration were also confirmed by the established computational resource CIBERSORT. Notably, TMPRSS2 was positively associated with the infiltration abundances of resting dendritic cells,

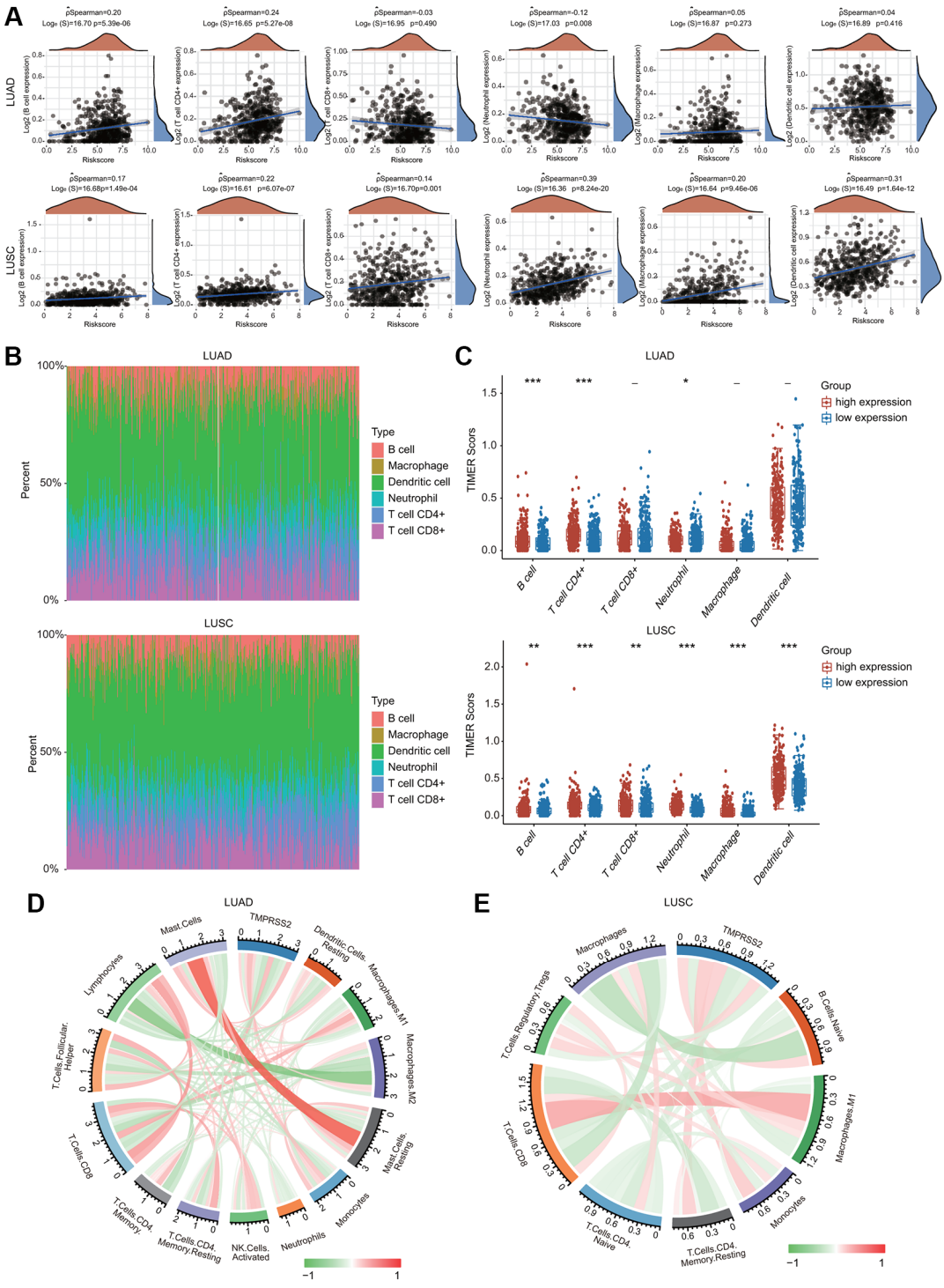

Figure 8. Association between TMPRSS2 expression and infiltration levels of immune cells in lung cancer. (**A**) TMPRSS2 expression was significantly correlated with the infiltration levels of various immune cells in LUAD and LUSC in the TIMER database. (**B**, **C**) TMPRSS2 expression was significantly associated with the infiltration of immune cells in LUAD and LUSC according to the CIBERSORT algorithm. (**D**) Heatmap of the correlation of TMPRSS2 and immune checkpoints in LUAD. (**E**) Heatmap of the correlation of TMPRSS2 and immune checkpoints in LUSC. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

dendritic cells, M2 macrophages, resting mast cells, mast cells, monocytes, and resting CD4 memory T cells but negatively associated with the infiltration abundances of lymphocytes, M0 macrophages, M1 macrophages, neutrophils, activated mast cells, activated NK cells, resting NK cells, plasma cells, activated memory CD4 T cells, CD8 T cells, follicular helper T cells and gamma delta T cells in LUAD (Figure 8D and Supplementary Figure 8A, 8B). Additionally, TMPRSS2 was positively associated with the infiltration abundances of naïve B cells, lymphocytes, resting mast cells, monocytes, Treg cells, neutrophils, and resting memory CD4 T cells but negatively correlated with the infiltration abundances of macrophages, M0 macrophages, M1 macrophages, CD8 T cells, naïve CD4 T cells, activated memory CD4 T cells, and eosinophils in LUSC (Figure 8E and Supplementary Figure 9A, 9B).

Correlations between TMPRSS2 and immune cell marker sets

To further explore the association between TMPRSS2 and these multiple populations of infiltrating immune cells, we established correlation between TMPRSS2 and different marker sets of multiple immune cells through the TIMER and GEPIA2 databases. TMPRSS2 mRNA expression was significantly correlated with most diverse immune cell markers in lung cancer (Tables 1 and 2).

Furthermore, we investigated the relationship between TMPRSS2 and various types of T cells. TMPRSS2 expression was strongly related with 37 of 54 T cell markers in LUAD and with 35 of 54 T cell markers in LUSC (Table 3). Significantly lower expression of CD274 (PD-1), PDCD-1 (PD-L1), PDCD1LG2, and LAG3 was observed in the TMPRSS2 high-expression group compared with the TMPRSS2 low-expression group in LUAD (Figure 9A). In contrast, higher expression of CTLA4, HAVCR2, PDCD1, TIGIT and SIGLEC15 was found in the TMPRSS2 high-expression group compared with the TMPRSS2 low-expression group in LUSC (Figure 9A). The correlations between TMPRSS2 and various immune checkpoints, including CTLA-4, PD-1 and PD-L1, were then assessed. TMPRSS2 expression was positively and strongly associated with ADORA2A, IL10RB, LAGLS9, TGFB1 and KDR but negatively associated with IDO1, LAG3, PD-1, PDCD1, PDCD1LG2, KIR2DL1 and KIR2DL3 in LUAD (Figure 9B). In contrast, TMPRSS2 expression was positively associated with most immune checkpoints in LUSC (Figure 9B). We also found that TMPRSS2 expression was significantly negatively correlated with TMB in LUAD and LUSC. Additionally, TMPRSS2 expression was weakly correlated with MSI in LUSC (Supplementary Figure 10A, 10B).

Prognostic analysis of TMPRSS2 expression according to immune cell infiltration in lung cancer

We also estimated whether TMPRSS2 influenced the prognosis of patients with lung cancer through its effects on immune cell infiltration. Prognostic analysis according to TMPRSS2 expression in patients stratified by populations of related immune cell subgroups was carried out. The low expression of TMPRSS2 in the LUAD patient cohorts with increased CD4+ T cells, increased macrophages, decreased NK T cells and increased Th2 cells was linked to worse prognosis (Figure 10B, 10D, 10E, 10H). In addition, a significant correlation between low TMPRSS2 expression and inferior prognosis was observed in the cohorts with either increased or decreased CD8+ T cells, B cells, Treg cells, and Th1 cells populations in LUAD (Figure 10A, 10C, 10F, 10G). Moreover, the high expression of TMPRSS2 in the LUSC patient cohorts with decreased B cells, CD4+ memory T cells, CD8+ T cells, Th1 cells and Th2 cells exhibited worse OS (Supplementary Figure 11A, 11B, 11C, 11G, 11H). In contrast, no significant correlations between TMPRSS2 expression and prognosis were observed in the cohorts with either increased or decreased macrophage, NK T cell, or Treg cell populations in LUSC patients (Supplementary Figure 11D, 11E, 11F). These findings suggest that TMPRSS2 expression affects the prognosis of patients with lung cancer partially through immune cell infiltration.

TMPRSS2 expression was downregulated during SARS-CoV infection

We then investigated the effect of coronavirus on TMPRSS2 expression. We collected four GEO databases, GSE33267, GSE47962, GSE45042 and GSE156544, to assess TMPRSS2 expression during SARS-CoV infection. TMPRSS2 expression was significantly reduced in Calu-3 cells and HAE cultures infected with SARS-CoV (Figure 11A). Additionally, TMPRSS2 expression was decreased in human bronchial epithelial cells infected with SARS-CoV2, although the difference was not significant difference (Figure 11A).

We also examined the protein expression level of TMPRSS2 in lung cancer using the UALCAN database. The protein level of TMPRSS2 was also much lower in lung cancer tissues than in normal lung tissues (Figure 11B). We also retrieved the IHC staining data from the HPA database. Normal lung tissues exhibited moderate TMPRSS2 staining, while TMPRSS2 could not be detected in most lung cancer tissues (Figure 11C). The results indicated that TMPRSS2 had significantly lower protein expression

Table 1. Correlations between TMPRSS2 and various gene markers of immune cells in TIMER.

Description	Gene markers	LUAD				LUSC			
		None		Purity		None		Purity	
		P	Cor	*P*	Cor	*P*	Cor	*P*	Cor
T cell	CD2	0.907	0.005	0.418	0.037	***	0.233	**	0.133
	CD3D	0.089	−0.075	0.198	−0.058	***	0.208	*	0.1
B cell (general)	CD3E	0.84	0.009	0.313	0.046	***	0.259	***	0.161
	CD19	0.285	0.047	0.0506	0.088	***	0.289	***	0.196
	CD79A	0.685	0.018	0.214	0.056	***	0.269	***	0.164
CD8+ T cell	CD8A	***	−0.154	**	−0.132	***	0.147	0.164	0.064
	CD8B	***	−0.156	**	−0.134	**	0.14	*	0.092
TAM	IL10	0.736	−0.015	0.889	0.006	***	0.277	***	0.193
	CD68	0.557	−0.026	0.992	0	***	0.251	**	0.143
Monocyte	CCL2	0.614	0.022	0.520	0.029	***	0.224	**	0.148
	CSF1R	**	0.12	***	0.152	***	0.312	***	0.206
	CD86	0.966	−0.002	0.512	0.03	***	0.3	***	0.196
M2	CD163	0.415	−0.036	0.77	−0.013	***	0.298	***	0.2
	MS4A4A	0.924	0.004	0.486	0.031	***	0.267	***	0.163
M1	VSIG4	0.825	−0.01	0.758	0.014	***	0.249	***	0.151
	PTGS2	0.265	0.049	0.352	0.042	***	0.266	***	0.223
	IRF5	*	0.097	**	0.12	0.897	−0.006	−0.024	0.598
Natural killer cell	KIR2DS4	*	−0.113	*	−0.116	**	0.129	*	0.104
	KIR3DL3	***	−0.234	***	−0.243	0.473	−0.032	0.280	−0.05
	KIR3DL2	***	−0.169	***	−0.173	**	0.126	0.0749	0.082
Neutrophils	KIR3DL1	*	−0.103	*	−0.104	**	0.141	*	0.093
	KIR2DL4	***	−0.391	***	−0.386	0.529	0.028	0.465	−0.034
	KIR2DL3	***	−0.229	***	−0.214	*	0.107	0.107	0.074
	KIR2DL1	**	−0.131	**	−0.13	**	0.124	*	0.09
	CCR7	***	0.233	***	0.295	***	0.339	***	0.26
	CEACAM8	***	0.391	***	0.394	***	0.175	***	0.172
	ITGAM	***	0.156	***	0.185	***	0.358	***	0.266
Dendritic cell	HLA-DPB1	***	0.307	***	0.358	***	0.358	***	0.266
	HLA-DRA	***	0.231	***	0.277	***	0.299	***	0.203
	HLA-DQB1	***	0.284	***	0.327	***	0.199	*	0.094
	HLA-DPA1	***	0.283	***	0.333	***	0.327	***	0.237
	CD1C	***	0.49	***	0.516	***	0.399	***	0.316
	NRP1	***	0.167	***	0.167	***	0.209	**	0.12
	ITGAX	0.0712	0.08	**	0.12	***	0.354	***	0.248

Table 2. Correlations between TMPRSS2 and various gene markers of immune cells in GEPIA2.

Description	Gene markers	LUAD		LUSC	
		P	Cor	*P*	Cor
TAM	CCL2	0.15	0.065	***	0.23
	CD68	0.51	0.03	***	0.24
	IL10	0.37	0.041	***	0.27
T cell (general)	CD2	0.81	0.011	***	0.23
	CD3D	0.077	−0.08	***	0.2
	CD3E	0.66	0.02	***	0.25
B cell	CD79A	0.68	−0.019	***	0.25
	CD19	0.59	0.025	***	0.26
CD8+ T cell	CD8A	**	−0.15	**	0.14
	CD8B	***	−0.15	**	0.13
Monocyte	CD86	0.44	0.035	***	0.29
	CSF1R	***	0.16	***	0.31

M1	PTGS2	0.092	0.077	***	0.27
	IRF5	**	0.13	0.78	−0.013
M2	VSIG4	0.52	0.029	***	0.24
	MS4A4A	0.28	0.049	***	0.26
	CD163	*	−0.097	***	0.27
Neutrophils	ITGAM	***	0.22	***	0.36
	CCR7	***	0.25	***	0.34
	CEACAM8	***	0.46	***	0.22
Natural killer cell	KIR2DS4	**	−0.14	**	0.12
	KIR3DL3	***	−0.25	0.93	0.0041
	KIR3DL2	***	−0.15	**	0.14
	KIR3DL1	*	−0.11	***	0.18
	KIR2DL4	***	−0.39	0.57	0.026
	KIR2DL3	***	−0.24	*	0.1
	KIR2DL1	***	−0.16	**	0.13
Dendritic cell	HLA-DQB1	***	0.23	**	0.12
	HLA-DPB1	***	0.33	***	0.36
	HLA-DRA	***	0.27	***	0.3
	HLA-DPA1	***	0.32	***	0.32
	ITGAX	**	0.12	***	0.34
	NRP1	***	0.22	***	0.24
	CD1C	***	0.5	***	0.4

Table 3. Correlations between TMPRSS2 and gene markers of diverse types of T cell in TIMER.

Description	Gene markers	LUAD				LUSC			
		None		Purity		None		Purity	
		P	Cor	*P*	Cor	*P*	Cor	*P*	Cor
Th1	TNF	***	0.173	**	0.124	0.9	0.006	0.128	−0.07
	IFNG	0.105	0.072	*	0.089	***	0.242	**	0.149
	TBX21	0.401	−0.037	0.807	−0.011	***	0.242	***	0.152
	STAT1	***	−0.222	***	−0.211	0.620	0.022	0.329	−0.045
	STAT4	*	0.101	**	0.13	***	0.341	***	0.249
Th1-like	HAVCR2	0.492	−0.03	0.907	−0.005	***	0.256	***	0.153
	IFNG	***	−0.323	***	−0.316	0.965	0.002	0.164	−0.064
	CXCR3	0.534	0.027	0.251	0.052	***	0.272	***	0.185
	CXCL13	*	−0.089	0.101	−0.074	***	0.171	0.131	0.69
	BHLHE40	***	0.156	**	0.142	***	0.301	***	0.257
	CD4	***	0.163	***	0.212	***	0.35	***	0.252
Th2	BCL6	***	0.303	***	0.304	***	0.175	***	0.225
	STAT5A	***	0.163	***	0.2	***	0.359	***	0.274
	GATA3	0.620	−0.022	0.784	−0.012	***	0.149	0.073	0.082
	STAT3	***	0.343	***	0.353	***	0.336	***	0.308
	STAT6	***	0.372	***	0.382	***	0.259	***	0.264
	IL13	0.0538	0.085	*	0.095	***	0.187	**	0.128
	IL21	***	−0.149	***	−0.137	**	0.142	0.1	0.075
	IL17A	0.0539	−0.085	0.182	−0.06	0.612	−0.023	0.0628	−0.085
Treg	STAT5B	***	0.237	***	0.247	0.256	0.051	0.201	0.059
	FOXP3	0.7	0.017	0.310	0.046	***	0.253	***	0.151
	TGFB1	***	0.266	***	0.291	**	0.143	0.131	0.069
	CCR8	0.128	0.067	*	0.096	***	0.254	**	0.164
Resting Treg	FOXP3	0.7	0.017	0.310	0.046	***	0.253	***	0.151
	IL2RA	**	−0.141	**	−0.135	***	0.206	*	0.102
Effector Treg T cell	FOXP3	0.7	0.017	0.310	0.046	***	0.253	***	0.151
	CTLA4	0.298	−0.046	0.594	−0.024	***	0.186	0.0992	0.076
	CCR8	0.128	0.067	*	0.096	***	0.254	**	0.164
	TNFRSF9	*	−0.109	*	−0.1	***	0.165	0.193	0.06

Effector T cell	FGFBP2	***	0.194	***	0.206	0.0532	−0.086	0.385	−0.04
	FCGR3A	**	−0.141	**	−0.122	***	0.187	0.0722	0.082
	CX3CR1	***	0.454	***	0.474	***	0.342	***	0.272
Naïve T cell	CCR7	***	0.233	***	0.295	***	0.339	***	0.26
	SELL	***	0.152	***	0.192	***	0.356	***	0.262
	TCF7	0.0732	0.079	*	0.097	***	0.197	**	0.136
	LEF1	0.236	0.052	0.195	0.058	*	−0.1	0.142	−0.067
	PDCD1	**	−0.126	*	−0.111	***	0.214	**	0.121
	DUSP4	***	−0.301	***	−0.296	***	0.174	*	0.113
	GZMK	0.843	0.009	0.350	0.042	***	0.23	**	0.134
	GZMA	***	−0.24	***	−0.234	*	0.106	0.761	0.014
	IFNG	***	−0.323	***	−0.316	0.965	0.002	0.164	−0.064
	CD69	**	0.133	***	0.177	***	0.295	***	0.198
	ITGAE	***	−0.199	***	−0.189	***	−0.166	*	−0.117
	CXCR6	0.0849	−0.076	0.254	−0.052	***	0.22	**	0.13
	MYADM	*	0.1	**	0.117	***	0.194	**	0.123
General memory T cell	CCR7	***	0.233	***	0.295	***	0.339	***	0.26
	SELL	***	0.152	***	0.192	***	0.356	***	0.262
	IL7R	**	0.126	***	0.156	***	0.255	***	0.152
Exhausted T cell	HAVCR2	0.492	−0.03	0.907	−0.005	***	0.256	***	0.153
	TIGIT	0.209	−0.055	0.494	−0.031	***	0.221	**	0.121
	LAG3	***	−0.211	***	−0.196	0.0521	0.087	0.947	0.003
	PDCD1	**	−0.126	*	0−0.11	***	0.214	**	0.121
	CXCL13	*	−0.089	0.101	−0.074	***	0.171	0.131	0.069
	LAYN	0.571	0.025	0.387	0.039	0.915	−0.005	0.661	−0.02

in lung cancer. These findings further confirmed the decreased expression of TMPRSS2 in lung cancer.

DISCUSSION

COVID-19 has proven to be a dangerous and far-reaching disease, and the number of infections and deaths worldwide continues to drastically rise worldwide [1–3]. Although most patients eventually recover, the world has not recently experienced another large-scale destruction in such a short period of time. The long-term effects of this virus are currently unclear, although it is thought that many patients will have serious sequelae from this infection. Thus, in addition to preventing infection, research on the factors that determine susceptibility to COVID-19 infection and the mechanisms behind these factors are critical for the control of SARS-CoV-2.

Lung cancer patients are at high risk of COVID-19 infection because the lung is the major target organ of SARS-CoV-2 infection [7]. COVID-19 appears to have a worse prognosis in cancer patients who have been admitted to the intensive care unit and in those requiring mechanical ventilation and increased mortality, especially in those who have recently received surgery or chemotherapy. SARS-CoV-2 infects humans by binding to ACE2, which is a transmembrane endopeptidase that can cleave angiotensin 1 and 2 and is expressed by epithelial cells of multiple organs, including the airway [1–5]. The cofactor that promotes SARS-CoV-2 infection is TMPRSS2, which could cleave the SARS-CoV-2 spike protein and possibly the protease furin. Additionally, TMPRSS2 expression is associated with the infectivity of various respiratory viruses. TMPRSS2-KO mice showed stronger resistance to influenza [17–19]. During the H1N1 epidemic in 2009, the TMPRSS2 variant that resulted in increased expression was associated with increased human susceptibility to influenza infection [20]. Previous study suggested that camostat, a serine and cysteine protease inhibitor of TMPRSS2, can partially but significantly block SARS-CoV infection, and the combination with alostatin (a cathepsin inhibitor) could significantly enhance the antiviral effect of camostat [21]. Understanding TMPRSS2 expression in lung cancer patients and its relationship with prognosis may help clarify why cancer patients are more likely to be infected with SARS-CoV-2 and help determine whether lung cancer immunotherapy may change susceptibility to SARS-CoV-2 infection.

When considering the harmful consequences of cancer and COVID-19 disease, an initial hypothesis is that the cancer tissue itself may have higher expression levels of related genes, allowing the virus to enter. However, we found that TMPRSS2 expression does not support this hypothesis in the present study. In contrast to our speculation, TMPRSS2 expression in lung cancer tissues was generally downregulated (Figures 1 and 11).

Nevertheless, in the present study, the GSEA results revealed that TMPRSS2 was associated with influenza A, herpes simplex virus 1 infection and Epstein–Barr virus infection, indicating that TMPRSS2 indeed plays a role in viral infection (Figure 7). Previous studies have shown that TMPRSS2 expression was significantly lower in nasopharyngeal swabs of SARS-CoV-2-infected patients than in those of healthy people and patients with other viral acute respiratory diseases [22]. Furthermore, the low TMPRSS2 expression predicted a short survival time in patients with lung cancer (Figure 3). We also evaluated the prognostic value of TMPRSS2 for lung cancer patients by performing Cox regression analyses and prognostic nomograms based on the correlation between TMPRSS2 expression and OS in lung cancer (Figure 4). These observations support that TMPRSS2 is related to carcinogenesis and may function as a promising candidate biomarker for predicting the prognosis of lung cancer. In fact, a pan-cancer analysis identified that both TMPRSS2 and ACE2 were commonly expressed at low levels in cancers compared with matched individuals [14]. A recent study using single-cell RNA-seq data demonstrated that TMPRSS2 is highly expressed in colorectal epithelial tissues and colorectal cancer [23]. More importantly, colorectal cancer patients with SARS-CoV-2 infection exhibited higher rates of lymphopenia, higher levels of hypersensitive C-reactive protein and a higher death rate than COVID-19 patients without colorectal cancer [23]. In contrast, TMPRSS2 expression was downregulated in head and neck cancer and oral squamous cell carcinoma [24–26]. Decreased TMPRSS2 expression was correlated with TP53 mutation and worse OS and DFS in head and

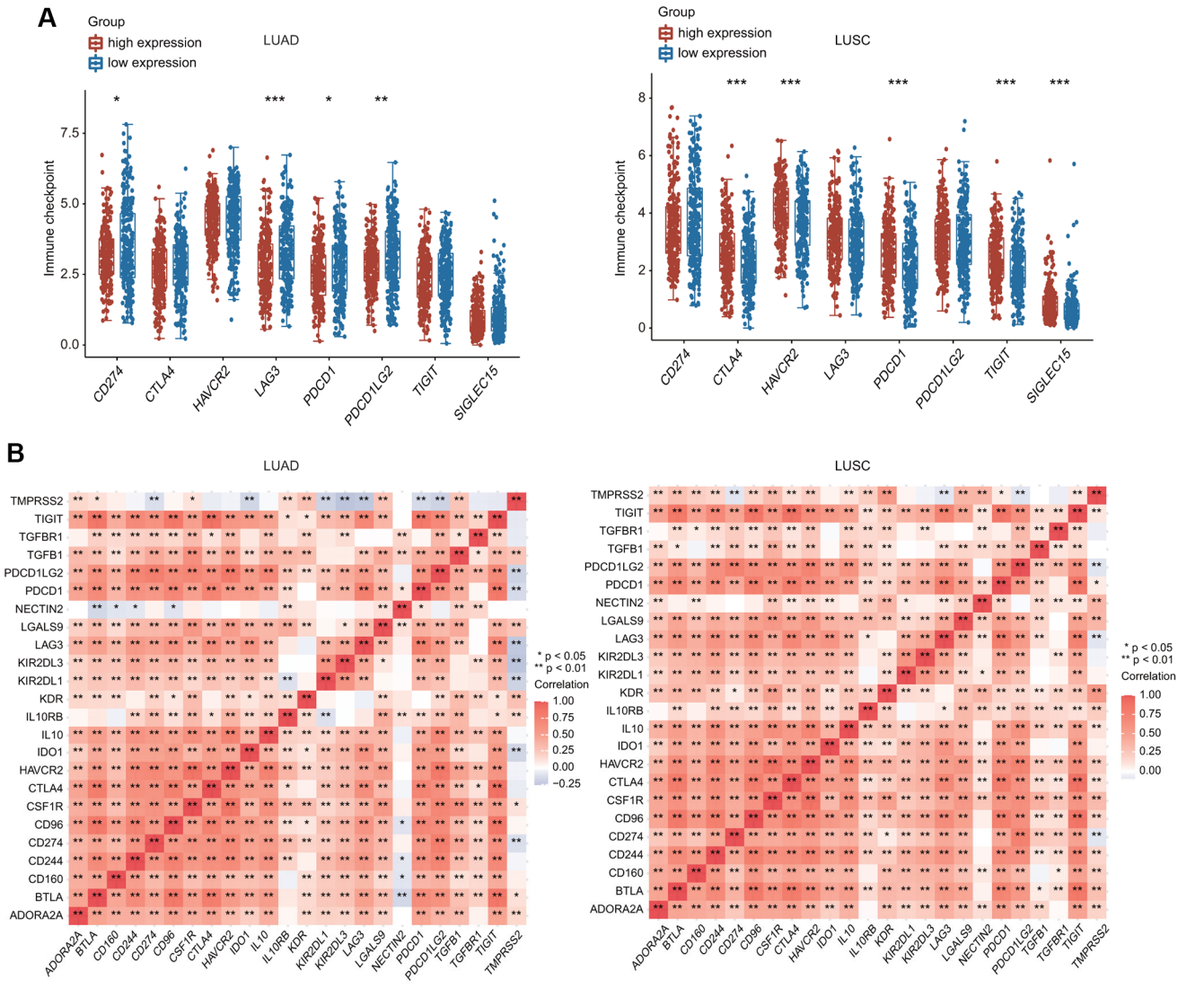

Figure 9. Relationship between TMPRSS2 expression and immune checkpoint genes. (**A**) The expression of multiple immune checkpoint genes between TMPRSS2 high-expression group and TMPRSS2 low-expression group in LUAD and LUSC. (**B**) Heatmap of correlations between TMPRSS2 expression and immune checkpoint genes in LUAD and LUSC.

neck cancer patients. Knockdown of mutant p53 greatly increased TMPRSS2 expression in head and neck cancer cells, indicating that p53 may modulate TMPRSS2 expression [24]. Moreover, a group of microRNAs was negatively associated with TMPRSS2 expression, indicating that TMPRSS2 expression may be regulated at the posttranscriptional level [24]. In addition, TMPRSS2 expression was also downregulated

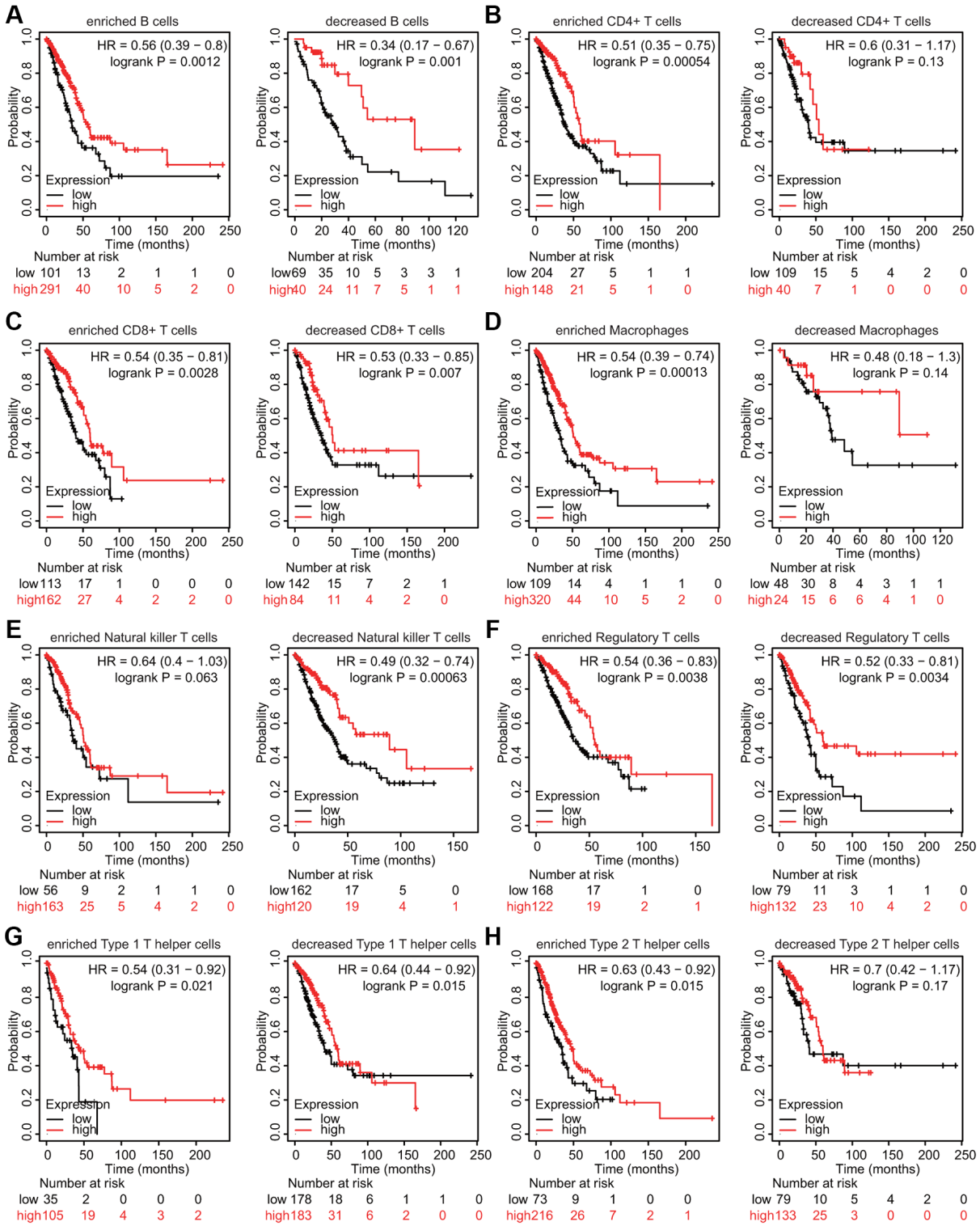

Figure 10. Kaplan–Meier survival curves based on high and low expression levels of TMPRSS2 in immune cell subgroups in LUAD. (**A**–**H**) The relationship between TMPRSS2 expression and the OS rate in different immune cell subgroups of LUAD patients was explored.

in tumor tissues in head and neck cancer patients with COVID-19 compared with matched normal individuals [24]. Accumulating evidence indicates that TMPRSS2 plays an important role in the oncogenesis of prostate cancer [27, 28]. Normally, TMPRSS2 is mainly expressed on the luminal side of the prostatic epithelium but is significantly upregulated in malignant prostatic cells and tissues [29]. Increased TMPRSS2 expression correlated with the poor survival of prostate cancer patients. TMPRSS2 promotes prostate oncogenesis not only through elevated expression but also though aberrant cellular localization that induces the loss of epithelial polarity [30]. High levels of TMPRSS2 also facilitate the tumor growth, progression, invasion and metastasis by modulating the activation of matriptase and the integrity of the ECM network. Moreover,

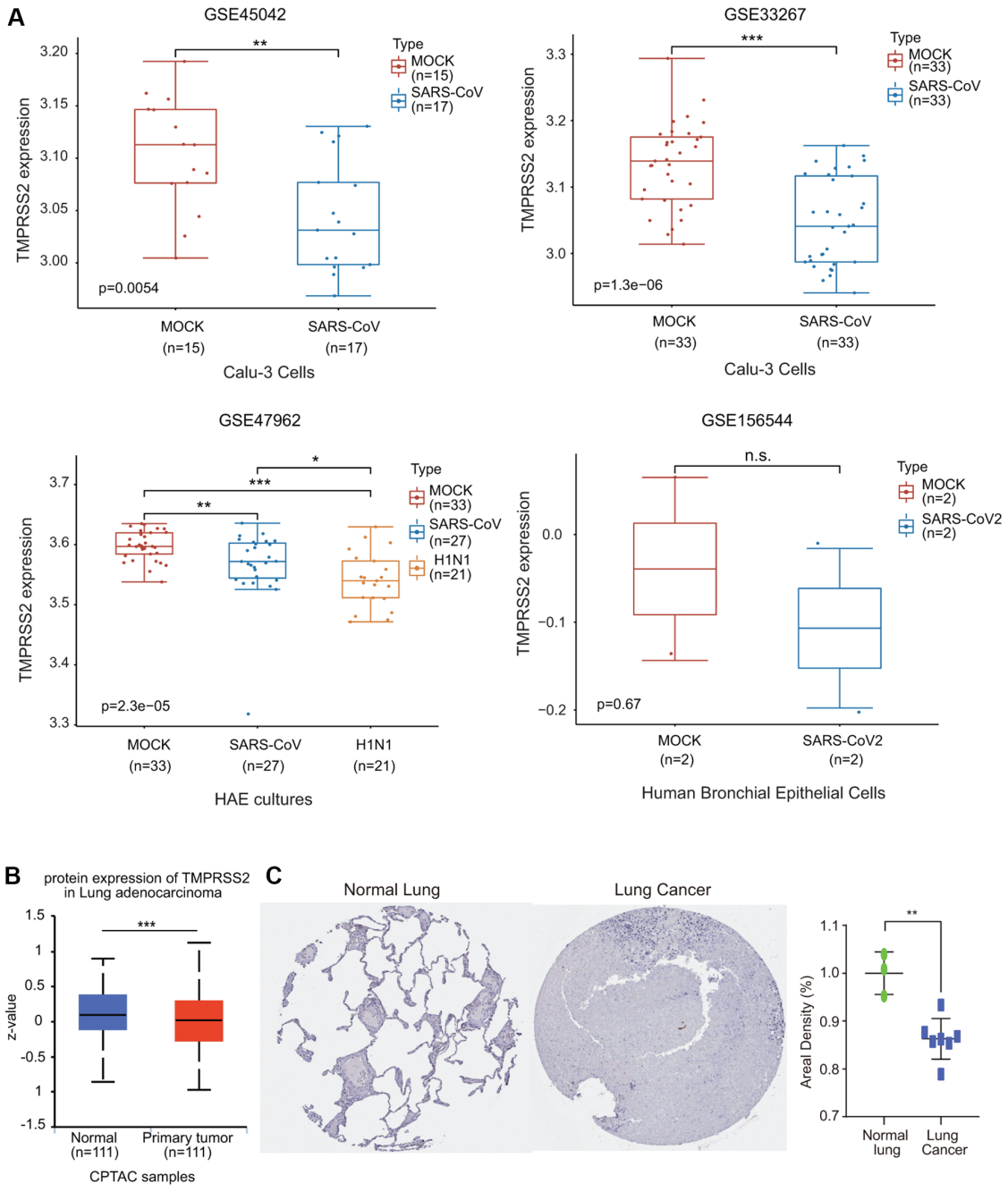

Figure 11. The alteration of TMPRSS2 expression during SARS-CoV-2 infection. (**A**) The change in TMPRSS2 expression in the GSE45042 (mock, *n* = 15; SARS-CoV, *n* = 17), GSE33267 (mock, *n* = 33; SARS-CoV, *n* = 33), GSE17962 (mock, *n* = 33; SARS-CoV, *n* = 27; H1N1, *n* = 21), and GSE156544 (mock, *n* = 2; SARS-CoV2, *n* = 2) datasets. (**B**) TMPRSS2 protein levels in lung cancer tissues (*n* = 111) and normal tissues (*n* = 111) from the UALCAN database. (**C**) TMPRSS2 protein level in lung cancer and normal tissues from the HPA database. The staining was quantified (normal lung tissue, *n* = 3; lung cancer, *n* = 7). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

inhibition of apoptosis was found in TMPRSS2-ERG-positive prostate cancer cells. This finding may be due to the destruction of the intracellular death domain and/or the corresponding receptor. A recent study identified HAI-2, a cognate inhibitor of TMPRSS2, as mediating the proteolytic activity of TMPRSS2 to inhibit the invasion and metastasis of prostate cancer [31]. In particular, the most common chromosomal aberration in prostate cancer is the fusion of erythroblast-specific-related gene (ERG) and the 5′-UTR of TMPRSS2 [32, 33]. Overexpression of ERG has been found in approximately 40%–50% of primary prostate cancers. Androgens and androgen receptors regulate TMPRSS2 and ACE2 expression [27, 28]. Men with higher androgen receptor transcriptional activity have a higher risk of TMPRSS2-ERG fusion-positive prostate cancer. In a study of 9280 COVID-19 patients from 68 hospitals in northeastern Italy, the researchers found that patients with prostate cancer and patients not treated with androgen deprivation were more susceptible to SARS-CoV-2 infection than patients treated with androgen deprivation, which would reduce the expression of TMPRSS2 [34]. Thus, prostate cancer patients with anti-AR treatment may be less susceptible to SARS-CoV-2 infection. Anti-AR therapy can be used as a therapeutic strategy and preventive option in patients with prostate cancer to inhibit the entry of viruses [35].

Based on our GO and KEGG analysis results, various metabolic pathways, such as fatty acid derivative metabolic process, icosanoid metabolic process, icosanoid biosynthetic process, leukotriene biosynthetic/metabolic process, leukotriene D4 biosynthetic/metabolic process, arachidonic acid metabolism, linolenic acid metabolism, glyoxylate and dicarboxylate metabolism, D-glutamine and D-glutamate metabolism, sulfur metabolism, and selenocompound metabolism, were significantly associated with TMPRSS2 in lung cancer (Figure 6). Consistent with the above findings, our GSEA results also suggest that TMPRSS2 may affect multiple metabolic processes, including fatty acid metabolic processes, lipid metabolic processes, butanoate metabolism, ether lipid metabolism, glycerophospholipid metabolism, and arachidonic acid metabolism (Figure 7). However, the relationships between TMRPSS2 and metabolism and SARS-CoV-2 infection are unclear and deserve further exploration.

COVID-19 also has a strong immune component, and its poor prognosis has recently been thought to be related to cytokine storms and the hyperinflammatory immune system [36, 37]. However, whether TMPRSS2 is involved in regulating antitumor immunity and its clinical significance in lung cancer remain unknown. Adaptive immunity after SARS-CoV-2 infection is necessary for effective virus clearance [38]. Because B and T cells respond quickly to infection and play a key role in defending against viral infection, systematic studies of changes in B and T cells in patients with COVID-19 will be important to reveal the immune response to SARS-COV-2 infection and will also provide insights for the diagnosis and treatment of COVID-19. In SARS-CoV-infected patients, the acute phase of infection was correlated with a severe reduction in the number of T cells in the blood, with a sharp reduction in the number of CD4 and CD8 T cells compared with that in healthy controls [39, 40]. These findings imply that SARS-CoV infection can impair cellular immunity early in the disease course. By analyzing blood samples from COVID-19 patients and healthy donors, it was demonstrated that TfH (follicular helper CD4 T cells) and GCB (germinal center B) cells were significantly increased in patients with mild or moderate symptoms, while patients with severe COVID-19 showed lymphocyte dysfunction characterized by the severe depletion of CD4+ lymphocytes and subsequent B-cell lymphopenia [41]. In addition, using single-cell RNA sequencing, CD8+ and CD4+ T cells were markedly decreased, while B cells were significantly increased during the recovery period of COVID-19 [41, 42]. Overall, these findings provide a preliminary understanding of the phenotypes of the T cell and B cell subtypes related to COVID-19.

In this study, KEGG and GO analyses and GSEA indicated that various immune-related pathways, such as myeloid leukocyte activation, leukocyte-mediated immunity, cytokine production, immune response-activating cell surface receptor signaling pathway, activation of the innate immune response, Th17 cell differentiation, cytokine–cytokine receptor interaction, and TNF signaling pathway, were significantly associated with TMPRSS2 expression (Figures 6 and 7). Considering the relationship between TMPRSS2 and the immune response, low TMPRSS2 expression in lung cancer patient tissues may lead to a decline in the immune function of patients with SARS-CoV-2 infection. Intriguingly, we observed that TMPRSS2 expression correlated with infiltrating levels of CD8+ T cells, B cells, CD4+ T cells, neutrophils, macrophages, and dendritic cells in lung cancer (Figure 8). Additionally, we found that TMPRSS2 was obviously associated with various gene set markers of different types of immune cells (Tables 1–3). According to the results of single-cell RNA-seq analysis, TMPRSS2 was expressed not only in colorectal epithelial cells but also in master cells, macrophages, B cells and T cells in colorectal cancer tissues [23]. This finding may be one of the reasons

why lung cancer patients are more likely to be infected with this novel coronavirus.

To confirm the change in TMPRSS2 expression after SARS-CoV-2 infection, we utilized four GEO datasets. The expression of TMPRSS2 in three datasets, GSE33267, GSE47962, and GSE45042, was significantly reduced in response to SARS-CoV infection (Figure 11). We also investigated the effect of SARS-CoV-2 infection on TMPRSS2 expression in Vero E6 cells. Although there was no significant difference, TMPRSS2 expression exhibited a decreasing trend (Figure 11). In fact, in the GSE156544 dataset, there were only two samples of SARS-CoV-2 infection. Because SARS-CoV-2 shares high homology with SARS-CoV, the TMPRSS2 expression level may similarly be reduced with SARS-CoV-2 infection. The downregulated expression of TMPRSS2 caused by SARS-CoV-2 infection may aggravate a variety of symptoms. Lung cancer patients should take adequate preventive measures to avoid COVID-19 infection and continuously monitor cell metabolism- and immune-related indicators [43].

In summary, we systematically analyzed the clinical significance and molecular mechanism of TMPRSS2 in lung cancer. TMPRSS2 expression was significantly downregulated in lung cancer. Decreased TMPRSS2 related with a poor prognosis and was associated with immune cell infiltration in lung cancer. The DNA methylation level of the TMPRSS2 promoters showed marked increases in LUAD and LUSC, indicating a potential cellular mechanism of TMPRSS2 gene expression in lung cancer. More importantly, TMPRSS2 expression was significantly decreased during SARS-CoV infection. Based on these results, we identified and elucidated the important roles of TMPRSS2 in lung cancer and the underlying mechanisms associated with its immune infiltration. However, there are several limitations. First, we did not perform *in vitro* or *in vivo* experiments to validate the precise roles and molecular mechanisms of TMPRSS2 in lung cancer. Further studies are required to confirm the prognostic value and mechanism by which TMPRSS2 influences the oncogenesis of lung cancer. Second, the present study lacks clinical information on lung cancer combined with SARS-CoV-2 infection data. Third, several variants in TMPRSS2 have been recently identified to affect the structure, function and stability of TMPRSS2. These variants may affect susceptibility to SARS-CoV-2 infection and lung cancer which needs to be confirmed in further studies. Although the lung is the primary target organ for COVID-19, it is necessary to identify TMPRSS2 expression in different cell types of lung which may affect the variable susceptibility to SARS-CoV-2 infection.

MATERIALS AND METHODS

Oncomine database analysis

The Oncomine database (http://www.oncomine.org) was used to determine TMPRSS2 expression in lung cancer tissues and adjacent corresponding normal tissues [44–48]. The investigation was carried out based on the following criteria: *P* value, <0.01; fold change, < −2.5; and gene ranking, all.

GEPIA2 database

We used GEPIA2 (http://gepia2.cancer-pku.cn/#index) to examine the mRNA expression level of TMPRSS2 in lung cancer and validate the correlation between TMPRSS2 and the expression levels of candidate genes [44–48].

UALCAN database

UALCAN (http://ualcan.path.uab.edu/), an online database containing transcriptome data from a variety of human cancers, was used to investigate the expression level and DNA methylation level of TMPRSS2 for comparisons not only between lung cancer and normal tissues but also across multiple subgroups stratified by clinicopathological parameters, such as sex, tumor stage, tumor grade and race.

TIMER database

The correlations between TMPRSS2 expression and the abundance of immune cell infiltrates in lung cancer datasets were analyzed using the TIMER database (https://cistrome.shinyapps.io/timer/) [44–48]. Correlations between TMPRSS2 expression and various gene marker sets of tumor-infiltrating immune cells were determined through a correlation module. The gene expression levels are represented as log2 TPM values.

cBioPortal database

The cBioPortal database enables users to investigate genomic profiles, such as the genetic alterations, survival curves and correlations of TMPRSS2 in lung cancer.

Kaplan–meier plotter analysis

The Kaplan–Meier plotter was applied to evaluate the prognostic value of TMPRSS2 in OS, FPS and PPS in lung cancer.

PrognoScan database

We used the PrognoScan database (http://www.prognoscan.org/), a comprehensive and user-friendly

database with clinical annotation, to assess the relationship between TMPRSS2 expression and prognostic information, including OS and RFS, in lung cancer patients. Cox *P* values and HRs with 95% confidence intervals were automatically calculated.

STRING and GeneMANIA databases analyses

GeneMANIA was applied to construct a gene–gene interaction network for TMPRSS2 in terms of physical interactions, coexpression, predictions, colocalization, and genetic interaction, as well as to evaluate their functions [44–48]. In addition, STRING database was used to develop a PPI network.

KEGG, GO and GSEA

KEGG and GO analyses were applied to examine the functions of TMPRSS2 in lung cancer. GO analysis was used to assess the biological processes (BP), molecular functions (MF) and cellular components (CC) related with TMPRSS2. We also applied GSEA to examine the potential mechanisms of TMPRSS2 in lung cancer. All of these analyses were performed using the R package ClusterProfiler [44–48].

CIBERSORT estimation

We used the CIBERSORT algorithm to identify the fractions of immune cells based on bulk samples from the LUAD and LUSC cohorts. The associations between TMPRSS2 expression and immune cell infiltration levels were evaluated using Spearman's correlation test.

IHC analysis

The TMPRSS2 protein expression in lung cancer and normal lung tissues from the HPA (Human Protein Atlas) database (https://www.proteinatlas.org/) were investigated by IHC staining.

SurvivalMeth

The SurvivalMeth online database was used to assess the DNA methylation of the TMPRSS2 gene and the influence of DNA methylation of TMPRSS2 on prognosis in LUAD and LUSC [44–48].

Cox regression analysis

Univariate and multivariate Cox regression analyses were carried out to evaluate the association between TMPRSS2 expression and OS of lung cancer patients using the TCGA database. The forest was generated to show the *P* value, HR and 95% CI of each clinicopathologic parameter through the R package "forestplot".

Construction and evaluation of a nomogram

Based on clinical characteristics, we generated a nomogram to predict the probability of OS using the R package "rms" (https://www.rdocumentation.org/packages/rms). The C-index was calculated to estimate the predictive accuracy. Calibration curves were plotted to compare the predicted OS with actual OS rates.

Open Targets platform

The Open Targets platform (http://www.targetvalidation.org) was used to identify the associations of TMPRSS2 and human diseases.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

Xiaopeng Liu, Bing Liu, Yanan Shang and Pengxiu Cao contributed equally to this article. Xiaopeng Liu, Bing Liu, Yanan Shang, Pengxiu Cao, Jiajie Hou, Fei Chen, Yumei Fan, and Ke Tan collected the data and performed the data analysis. Ke Tan designed the study and wrote the manuscript. Yumei Fan and Ke Tan revised the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this study.

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REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, et al, and China Novel Coronavirus Investigating and Research Team. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020; 382:727–33.

https://doi.org/10.1056/NEJMoa2001017
PMID:31978945

2. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020; 395:497–506. https://doi.org/10.1016/S0140-6736(20)30183-5 PMID:31986264

3. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 579:270–3. https://doi.org/10.1038/s41586-020-2012-7 PMID:32015507

4. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, et al. Addendum: A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020; 588:E6. https://doi.org/10.1038/s41586-020-2951-z PMID:33199918

5. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, Schiergens TS, Herrler G, Wu NH, Nitsche A, Müller MA, Drosten C, Pöhlmann S. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020; 181:271–80.e8. https://doi.org/10.1016/j.cell.2020.02.052 PMID:32142651

6. Stopsack KH, Mucci LA, Antonarakis ES, Nelson PS, Kantoff PW. *TMPRSS2* and COVID-19: Serendipity or Opportunity for Intervention? Cancer Discov. 2020; 10:779–82. https://doi.org/10.1158/2159-8290.CD-20-0451 PMID:32276929

7. Bertram S, Heurich A, Lavender H, Gierer S, Danisch S, Perin P, Lucas JM, Nelson PS, Pöhlmann S, Soilleux EJ. Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. PLoS One. 2012; 7:e35876. https://doi.org/10.1371/journal.pone.0035876 PMID:22558251

8. Strope JD, Chau CH, Figg WD. TMPRSS2: Potential Biomarker for COVID-19 Outcomes. J Clin Pharmacol. 2020; 60:801–7. https://doi.org/10.1002/jcph.1641 PMID:32437018

9. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, Liu L, Shan H, Lei CL, Hui DSC, Du B, Li LJ, Zeng G, et al, and China Medical Treatment Expert Group for Covid-19. Clinical Characteristics of Coronavirus Disease 2019 in China. N Engl J Med. 2020; 382:1708–20. https://doi.org/10.1056/NEJMoa2002032 PMID:32109013

10. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68:394–424. https://doi.org/10.3322/caac.21492 PMID:30207593

11. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nature. 2018; 553:446–54. https://doi.org/10.1038/nature25183 PMID:29364287

12. Kong Q, Xiang Z, Wu Y, Gu Y, Guo J, Geng F. Analysis of the susceptibility of lung cancer patients to SARS-CoV-2 infection. Mol Cancer. 2020; 19:80. https://doi.org/10.1186/s12943-020-01209-2 PMID:32345328

13. Liang W, Guan W, Chen R, Wang W, Li J, Xu K, Li C, Ai Q, Lu W, Liang H, Li S, He J. Cancer patients in SARS-CoV-2 infection: a nationwide analysis in China. Lancet Oncol. 2020; 21:335–7. https://doi.org/10.1016/S1470-2045(20)30096-6 PMID:32066541

14. Bao R, Hernandez K, Huang L, Luke JJ. *ACE2* and *TMPRSS2* expression by clinical, HLA, immune, and microbial correlates across 34 human cancers and matched normal tissues: implications for SARS-CoV-2 COVID-19. J Immunother Cancer. 2020; 8:e001020. https://doi.org/10.1136/jitc-2020-001020 PMID:32675312

15. Dai M, Liu D, Liu M, Zhou F, Li G, Chen Z, Zhang Z, You H, Wu M, Zheng Q, Xiong Y, Xiong H, Wang C, et al. Patients with Cancer Appear More Vulnerable to SARS-CoV-2: A Multicenter Study during the COVID-19 Outbreak. Cancer Discov. 2020; 10:783–91. https://doi.org/10.1158/2159-8290.CD-20-0422 PMID:32345594

16. Wang R, Simoneau CR, Kulsuptrakul J, Bouhaddou M, Travisano KA, Hayashi JM, Carlson-Stevermer J, Zengel JR, Richards CM, Fozouni P, Oki J, Rodriguez L, Joehnk B, et al. Genetic Screens Identify Host Factors for SARS-CoV-2 and Common Cold Coronaviruses. Cell. 2021; 184:106–19.e14. https://doi.org/10.1016/j.cell.2020.12.004 PMID:33333024

17. Sakai K, Ami Y, Tahara M, Kubota T, Anraku M, Abe M, Nakajima N, Sekizuka T, Shirato K, Suzaki Y, Ainai A, Nakatsu Y, Kanou K, et al. The host protease

TMPRSS2 plays a major role in in vivo replication of emerging H7N9 and seasonal influenza viruses. J Virol. 2014; 88:5608–16.
https://doi.org/10.1128/JVI.03677-13
PMID:24600012

18. Iwata-Yoshikawa N, Okamura T, Shimizu Y, Hasegawa H, Takeda M, Nagata N. TMPRSS2 Contributes to Virus Spread and Immunopathology in the Airways of Murine Models after Coronavirus Infection. J Virol. 2019; 93:e01815–8.
https://doi.org/10.1128/JVI.01815-18
PMID:30626688

19. Tarnow C, Engels G, Arendt A, Schwalm F, Sediri H, Preuss A, Nelson PS, Garten W, Klenk HD, Gabriel G, Böttcher-Friebertshäuser E. TMPRSS2 is a host factor that is essential for pneumotropism and pathogenicity of H7N9 influenza A virus in mice. J Virol. 2014; 88:4744–51.
https://doi.org/10.1128/JVI.03799-13
PMID:24522916

20. Cheng Z, Zhou J, To KK, Chu H, Li C, Wang D, Yang D, Zheng S, Hao K, Bossé Y, Obeidat M, Brandsma CA, Song YQ, et al. Identification of TMPRSS2 as a Susceptibility Gene for Severe 2009 Pandemic A(H1N1) Influenza and A(H7N9) Influenza. J Infect Dis. 2015; 212:1214–21.
https://doi.org/10.1093/infdis/jiv246
PMID:25904605

21. Hoffmann M, Hofmann-Winkler H, Smith JC, Krüger N, Arora P, Sørensen LK, Søgaard OS, Hasselstrøm JB, Winkler M, Hempel T, Raich L, Olsson S, Danov O, et al. Camostat mesylate inhibits SARS-CoV-2 activation by TMPRSS2-related proteases and its metabolite GBPA exerts antiviral activity. EBioMedicine. 2021; 65:103255.
https://doi.org/10.1016/j.ebiom.2021.103255
PMID:33676899

22. Cao W, Feng Q, Wang X. Computational analysis of TMPRSS2 expression in normal and SARS-CoV-2-infected human tissues. Chem Biol Interact. 2021; 346:109583.
https://doi.org/10.1016/j.cbi.2021.109583
PMID:34284028

23. Liu C, Wang K, Zhang M, Hu X, Hu T, Liu Y, Hu Q, Wu S, Yue J. High expression of ACE2 and TMPRSS2 and clinical characteristics of COVID-19 in colorectal cancer patients. NPJ Precis Oncol. 2021; 5:1.
https://doi.org/10.1038/s41698-020-00139-y
PMID:33479506

24. Sacconi A, Donzelli S, Pulito C, Ferrero S, Spinella F, Morrone A, Rigoni M, Pimpinelli F, Ensoli F, Sanguineti G, Pellini R, Agrawal N, Izumchenko E, et al. TMPRSS2, a SARS-CoV-2 internalization protease is downregulated in head and neck cancer patients. J Exp Clin Cancer Res. 2020; 39:200.
https://doi.org/10.1186/s13046-020-01708-6
PMID:32967703

25. Zheng T, Yue P, Han T, Zhang K, Jiang Y, Wang S, Jiang L, Zhao B, Zhang X, Yan X. Identification of key factors shaping integrated levels of ACE2 and TMPRSS2 expression in head and neck squamous cell carcinoma. Front Biosci (Landmark Ed). 2021; 26:740–51.
https://doi.org/10.52586/4984
PMID:34719202

26. Sapkota D, Sharma S, Søland TM, Braz-Silva PH, Teh MT. Expression profile of SARS-CoV-2 cellular entry proteins in normal oral mucosa and oral squamous cell carcinoma. Clin Exp Dent Res. 2021. [Epub ahead of print].
https://doi.org/10.1002/cre2.510
PMID:34726347

27. Ko CJ, Huang CC, Lin HY, Juan CP, Lan SW, Shyu HY, Wu SR, Hsiao PW, Huang HP, Shun CT, Lee MS. Androgen-Induced TMPRSS2 Activates Matriptase and Promotes Extracellular Matrix Degradation, Prostate Cancer Cell Invasion, Tumor Growth, and Metastasis. Cancer Res. 2015; 75:2949–60.
https://doi.org/10.1158/0008-5472.CAN-14-3297
PMID:26018085

28. Lucas JM, Heinlein C, Kim T, Hernandez SA, Malik MS, True LD, Morrissey C, Corey E, Montgomery B, Mostaghel E, Clegg N, Coleman I, Brown CM, et al. The androgen-regulated protease TMPRSS2 activates a proteolytic cascade involving components of the tumor microenvironment and promotes prostate cancer metastasis. Cancer Discov. 2014; 4:1310–25.
https://doi.org/10.1158/2159-8290.CD-13-1010
PMID:25122198

29. Wilson S, Greer B, Hooper J, Zijlstra A, Walker B, Quigley J, Hawthorne S. The membrane-anchored serine protease, TMPRSS2, activates PAR-2 in prostate cancer cells. Biochem J. 2005; 388:967–72.
https://doi.org/10.1042/BJ20041066
PMID:15537383

30. Lucas JM, True L, Hawley S, Matsumura M, Morrissey C, Vessella R, Nelson PS. The androgen-regulated type II serine protease TMPRSS2 is differentially expressed and mislocalized in prostate adenocarcinoma. J Pathol. 2008; 215:118–25.
https://doi.org/10.1002/path.2330
PMID:18338334

31. Ko CJ, Hsu TW, Wu SR, Lan SW, Hsiao TF, Lin HY, Lin HH, Tu HF, Lee CF, Huang CC, Chen MM, Hsiao PW, Huang HP, Lee MS. Inhibition of TMPRSS2 by HAI-2 reduces prostate cancer cell invasion and metastasis. Oncogene. 2020; 39:5950–63.

https://doi.org/10.1038/s41388-020-01413-w
PMID:32778768

32. Tomlins SA, Laxman B, Varambally S, Cao X, Yu J, Helgeson BE, Cao Q, Prensner JR, Rubin MA, Shah RB, Mehra R, Chinnaiyan AM. Role of the TMPRSS2-ERG gene fusion in prostate cancer. Neoplasia. 2008; 10:177–88. https://doi.org/10.1593/neo.07822 PMID:18283340

33. Sun C, Dobi A, Mohamed A, Li H, Thangapazham RL, Furusato B, Shaheduzzaman S, Tan SH, Vaidyanathan G, Whitman E, Hawksworth DJ, Chen Y, Nau M, et al. TMPRSS2-ERG fusion, a common genomic alteration in prostate cancer activates C-MYC and abrogates prostate epithelial differentiation. Oncogene. 2008; 27:5348–53. https://doi.org/10.1038/onc.2008.183 PMID:18542058

34. Montopoli M, Zumerle S, Vettor R, Rugge M, Zorzi M, Catapano CV, Carbone GM, Cavalli A, Pagano F, Ragazzi E, Prayer-Galetti T, Alimonti A. Androgen-deprivation therapies for prostate cancer and risk of infection by SARS-CoV-2: a population-based study (N = 4532). Ann Oncol. 2020; 31:1040–5. https://doi.org/10.1016/j.annonc.2020.04.479 PMID:32387456

35. Cheng J, Zhou J, Fu S, Fu J, Zhou B, Chen H, Fu J, Wei C. Prostate adenocarcinoma and COVID-19: The possible impacts of TMPRSS2 expressions in susceptibility to SARS-CoV-2. J Cell Mol Med. 2021; 25:4157–65. https://doi.org/10.1111/jcmm.16385 PMID:33609069

36. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. Science. 2020; 368:473–4. https://doi.org/10.1126/science.abb8925 PMID:32303591

37. Takahashi T, Ellingson MK, Wong P, Israelow B, Lucas C, Klein J, Silva J, Mao T, Oh JE, Tokuyama M, Lu P, Venkataraman A, Park A, et al, and Yale IMPACT Research Team. Sex differences in immune responses that underlie COVID-19 disease outcomes. Nature. 2020; 588:315–20. https://doi.org/10.1038/s41586-020-2700-3 PMID:32846427

38. Yang X, Dai T, Zhou X, Qian H, Guo R, Lei L, Zhang X, Zhang D, Shi L, Cheng Y, Hu J, Guo Y, Zhang B. Naturally activated adaptive immunity in COVID-19 patients. J Cell Mol Med. 2020; 24:12457–63. https://doi.org/10.1111/jcmm.15771 PMID:32975374

39. Westmeier J, Paniskaki K, Karaköse Z, Werner T, Sutter K, Dolff S, Overbeck M, Limmer A, Liu J, Zheng X, Brenner T, Berger MM, Witzke O, et al. Erratum for Westmeier et al., "Impaired Cytotoxic CD8$^+$ T Cell Response in Elderly COVID-19 Patients". mBio. 2020; 11:e02805–20. https://doi.org/10.1128/mBio.02805-20 PMID:33173009

40. Yang X, Dai T, Zhou X, Qian H, Guo R, Lei L, Zhang X, Zhang D, Shi L, Cheng Y, Hu J, Guo Y, Zhang B. Analysis of adaptive immune cell populations and phenotypes in the patients infected by SARS-CoV-2. medRxiv. 2020. [Epub ahead of print]. https://doi.org/10.1101/2020.03.23.20040675

41. Zhang JY, Wang XM, Xing X, Xu Z, Zhang C, Song JW, Fan X, Xia P, Fu JL, Wang SY, Xu RN, Dai XP, Shi L, et al. Single-cell landscape of immunological responses in patients with COVID-19. Nat Immunol. 2020; 21:1107–18. https://doi.org/10.1038/s41590-020-0762-x PMID:32788748

42. Meckiff BJ, Ramírez-Suástegui C, Fajardo V, Chee SJ, Kusnadi A, Simon H, Eschweiler S, Grifoni A, Pelosi E, Weiskopf D, Sette A, Ay F, Seumois G, et al. Imbalance of Regulatory and Cytotoxic SARS-CoV-2-Reactive CD4$^+$ T Cells in COVID-19. Cell. 2020; 183:1340–53.e16. https://doi.org/10.1016/j.cell.2020.10.001 PMID:33096020

43. Liang X, Chen Y, Fan Y. Bioinformatics approach to identify common gene signatures of patients with coronavirus 2019 and lung adenocarcinoma. Environ Sci Pollut Res Int. 2021. [Epub ahead of print]. https://doi.org/10.1007/s11356-021-17321-9 PMID:34775559

44. Chen F, Fan Y, Hou J, Liu B, Zhang B, Shang Y, Chang Y, Cao P, Tan K. Integrated analysis identifies TfR1 as a prognostic biomarker which correlates with immune infiltration in breast cancer. Aging (Albany NY). 2021; 13:21671–99. https://doi.org/10.18632/aging.203512 PMID:34518441

45. Liu B, Fan Y, Song Z, Han B, Meng Y, Cao P, Tan K. Identification of DRP1 as a prognostic factor correlated with immune infiltration in breast cancer. Int Immunopharmacol. 2020; 89:107078. https://doi.org/10.1016/j.intimp.2020.107078 PMID:33049497

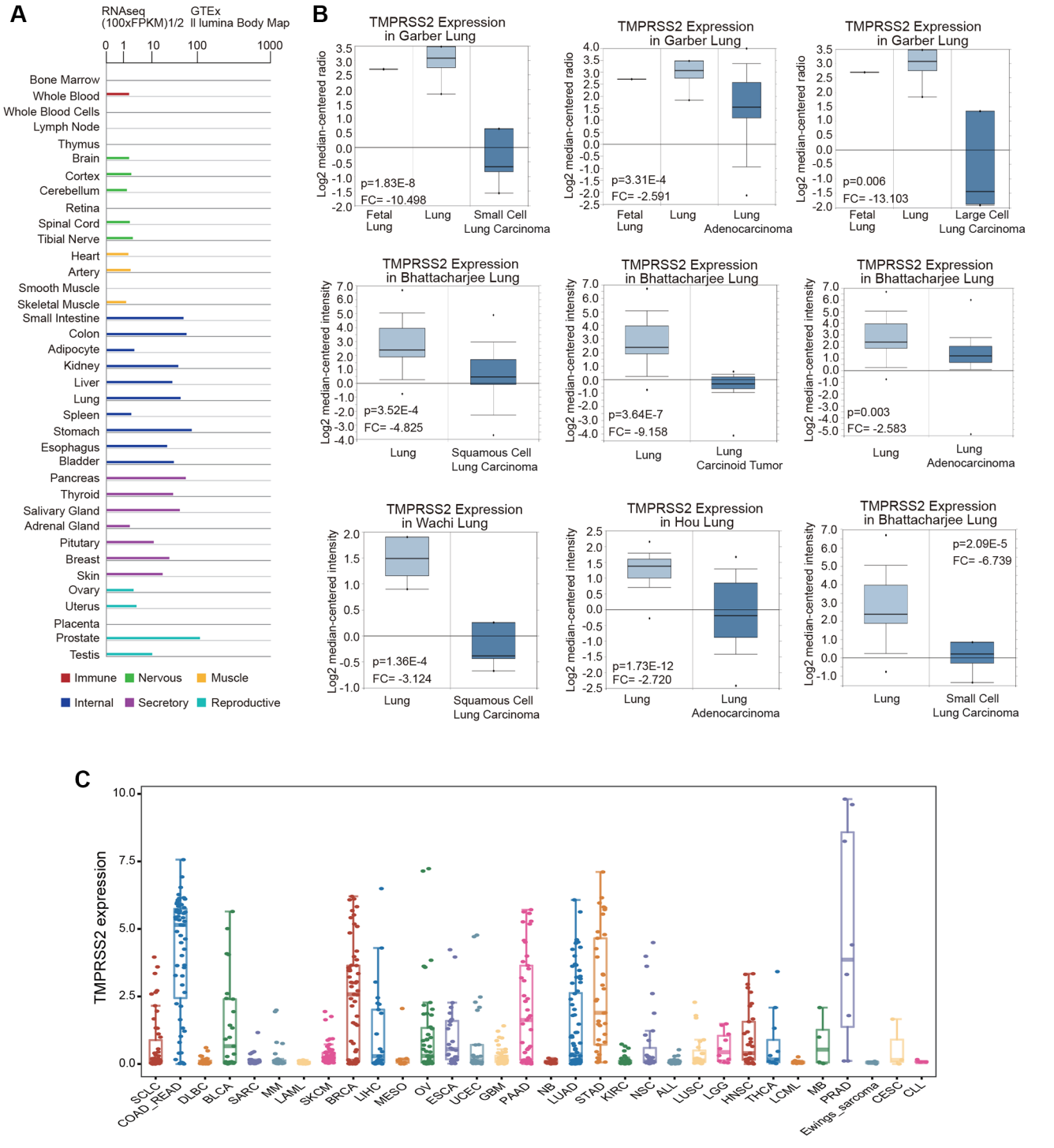
46. Chen F, Han B, Meng Y, Han Y, Liu B, Zhang B, Chang Y, Cao P, Fan Y, Tan K. Ceruloplasmin correlates with immune infiltration and serves as a prognostic biomarker in breast cancer. Aging (Albany NY). 2021; 13:20438–67. https://doi.org/10.18632/aging.203427 PMID:34413268

47. Chen F, Fan Y, Cao P, Liu B, Hou J, Zhang B, Tan K. Pan-Cancer Analysis of the Prognostic and Immunological Role of HSF1: A Potential Target for Survival and Immunotherapy. Oxid Med Cell Longev. 2021; 2021:5551036.
https://doi.org/10.1155/2021/5551036
PMID:34239690

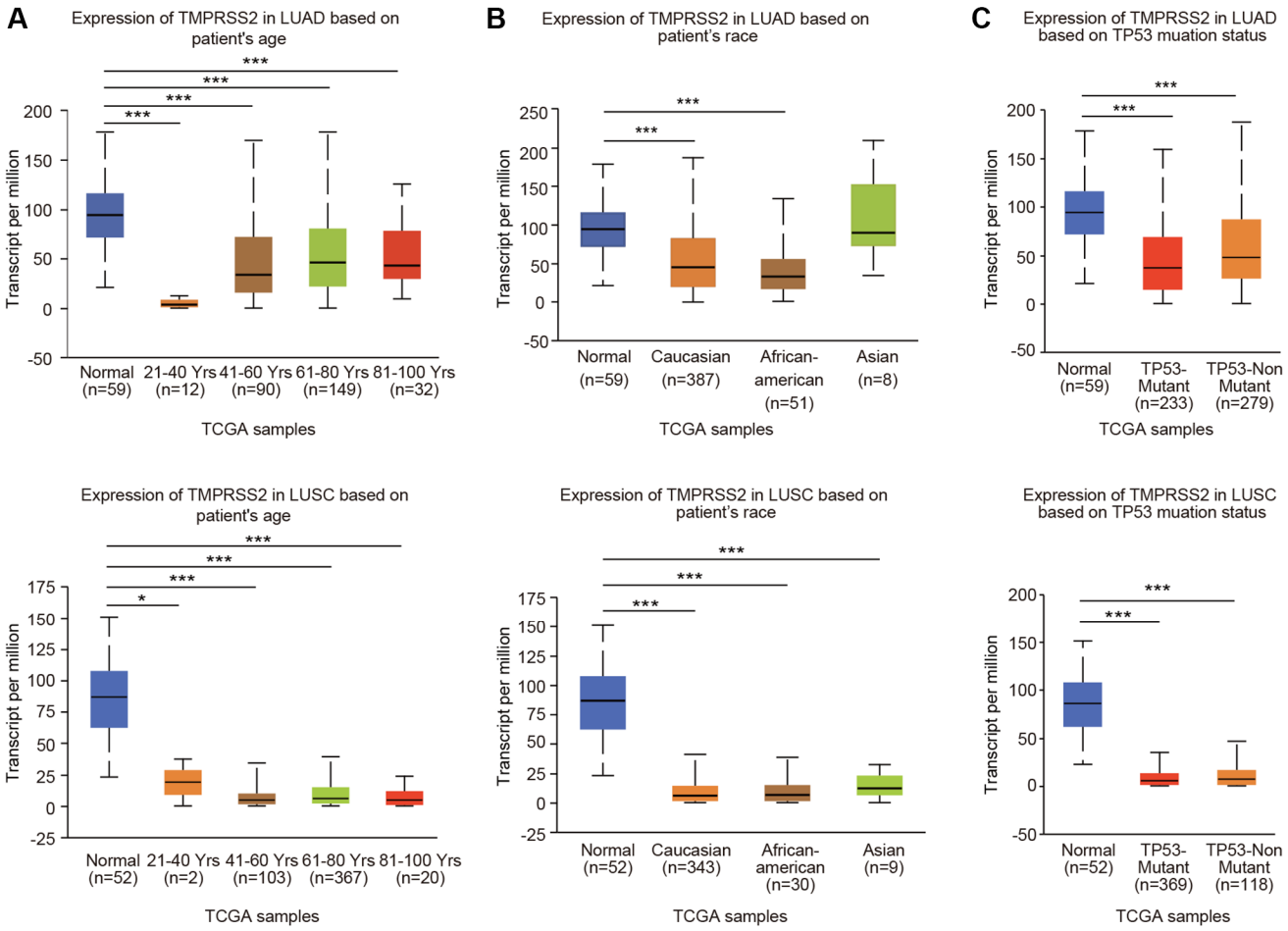
48. Fan Y, Liu B, Chen F, Song Z, Han B, Meng Y, Hou J, Cao P, Chang Y, Tan K. Hepcidin Upregulation in Lung Cancer: A Potential Therapeutic Target Associated With Immune Infiltration. Front Immunol. 2021; 12:612144.
https://doi.org/10.3389/fimmu.2021.612144
PMID:33868231

SUPPLEMENTARY MATERIALS

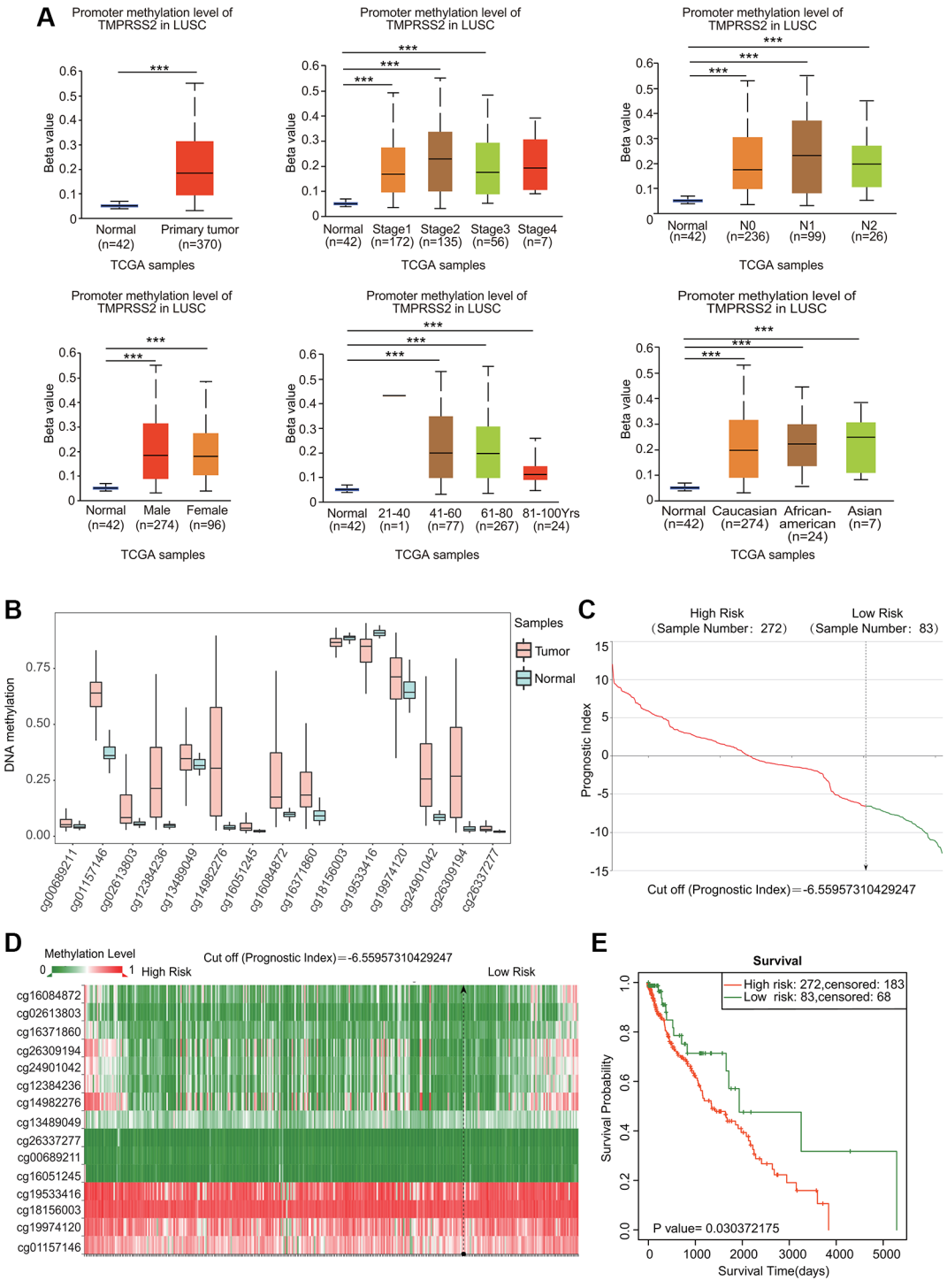
Supplementary Figures



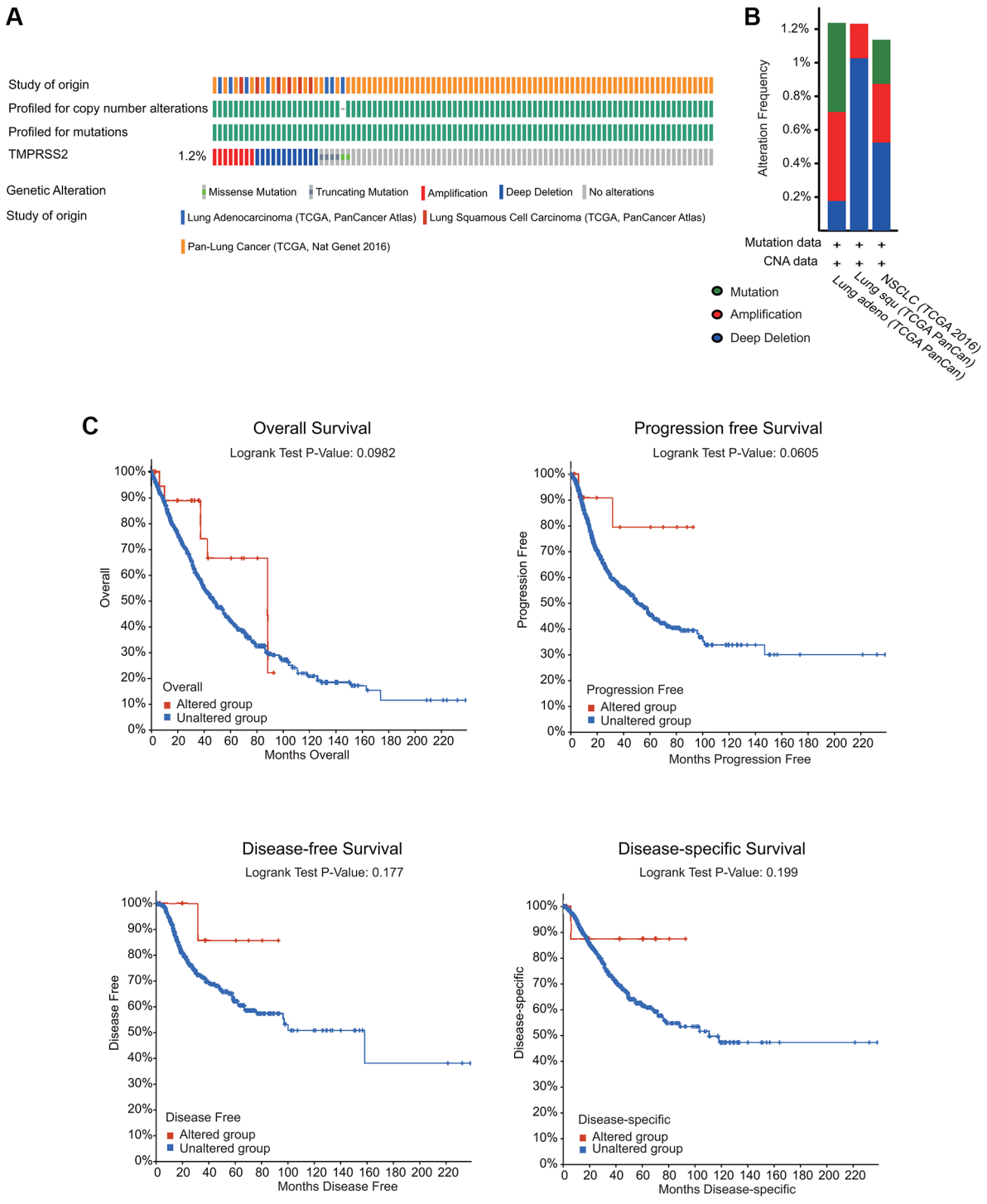
Supplementary Figure 1. (**A**) TMPRSS2 expression in different organs and tissues. (**B**) TMPRSS2 expression in different types of lung cancer patients and normal individuals from the Oncomine database. (**C**) TMPRSS2 expression in different types of cancer cells using the CCLE database.



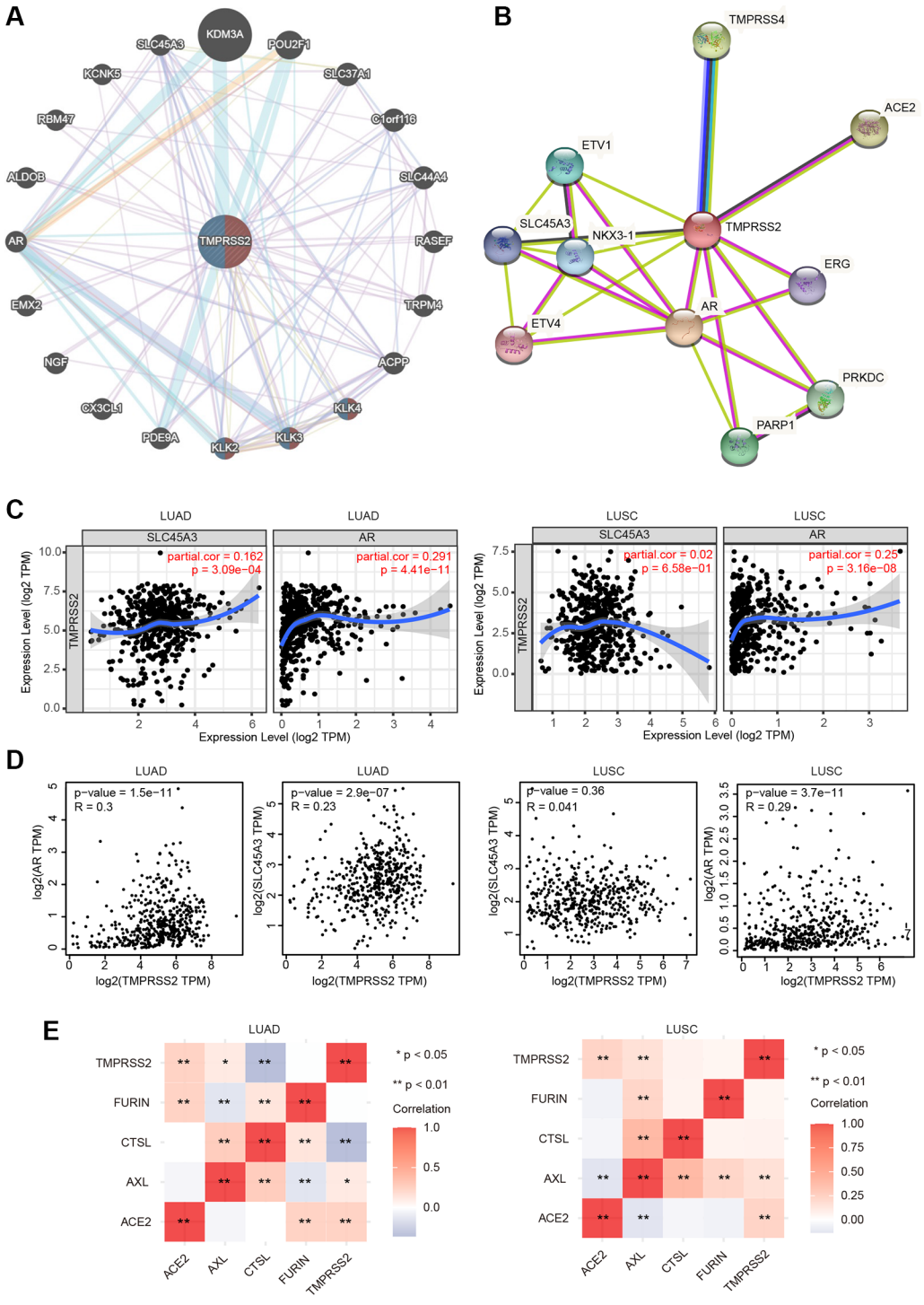
Supplementary Figure 2. TMPRSS2 expression was assessed in (**A**) patients with different ages, (**B**) patients with different races, (**C**) patients with different TP53 statuses from the UALCAN database.



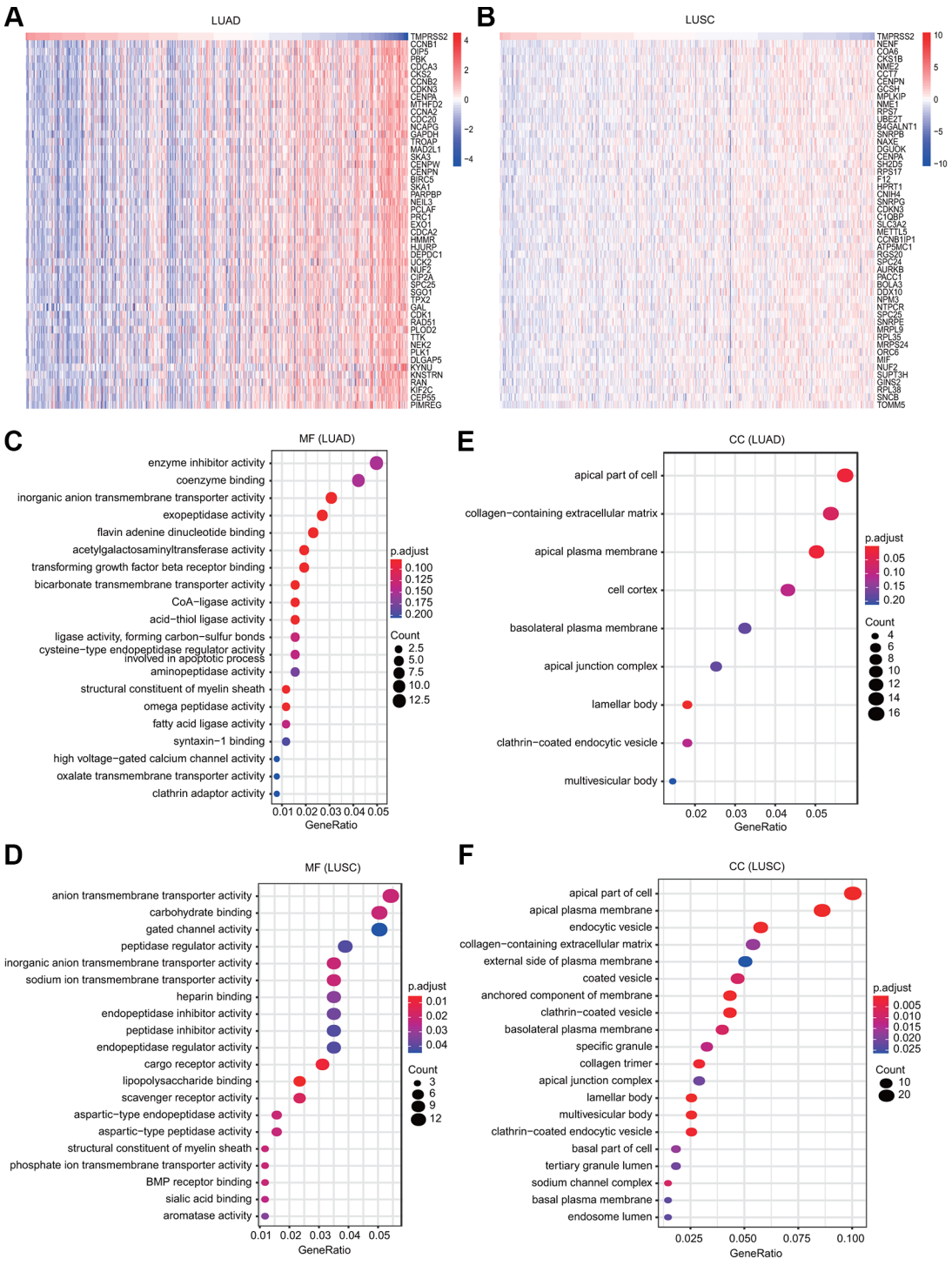
Supplementary Figure 3. DNA methylation of TMPRSS2 in LUSC. (**A**) Association of DNA methylation of TMPRSS2 with clinicopathological parameters of LUSC. (**B**) Methylation levels of TMPRSS2 in LUSC according to the SurvivalMeth database. (**C**) The distribution of prognostic index in LUSC. (**D**) The heatmap of DNA methylation of TMPRSS2 in LUSC. (**E**) The prognostic potential of DNA methylation of TMPRSS2 in LUSC based on the SurvivalMeth database.



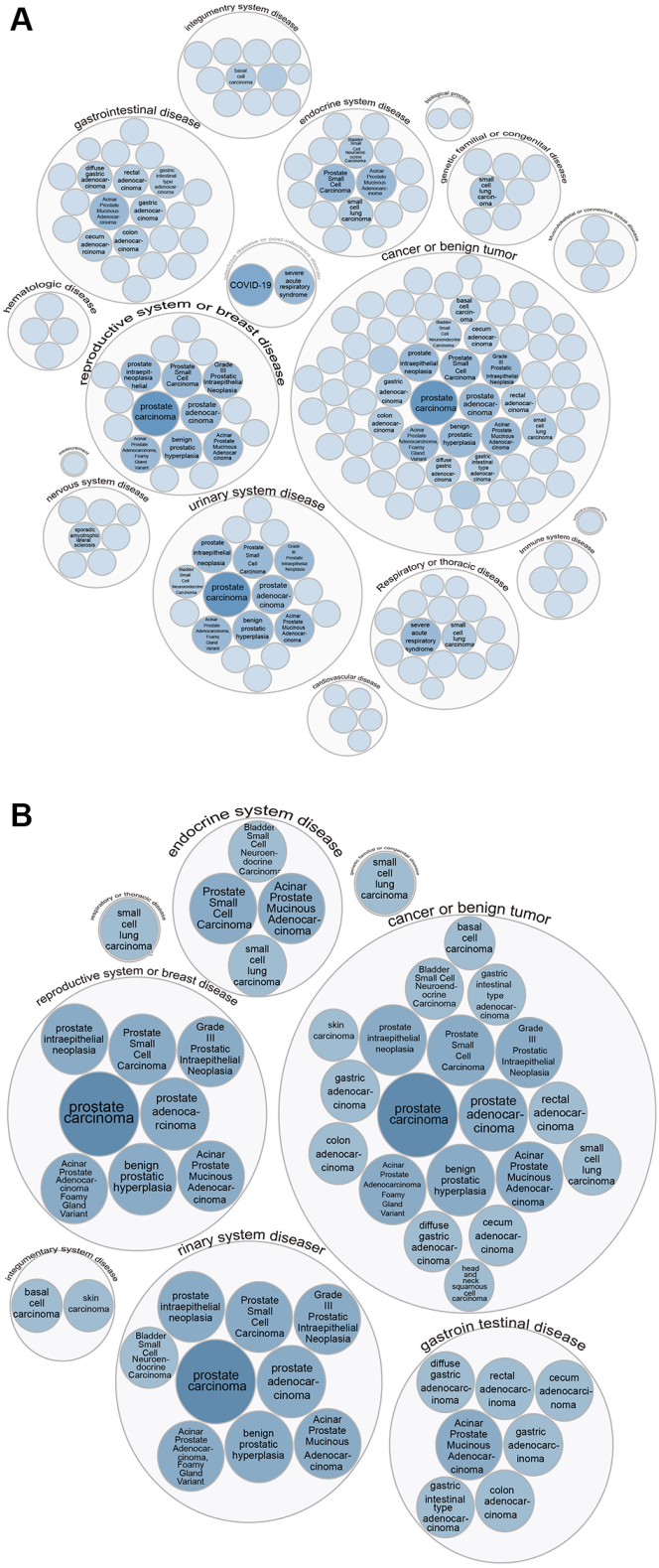
Supplementary Figure 4. Alteration frequency of TMPRSS2. (**A**) OncoPrint visual summary of alterations on a query of TMPRSS2 from the cBioPortal database. (**B**) Summary of TMPRSS2 genetic alterations in lung cancer. (**C**) Kaplan-Meier plots comparing OS, PFS, DFS and DSS in cases with or without TMPRSS2 gene alterations from the cBioPortal database.



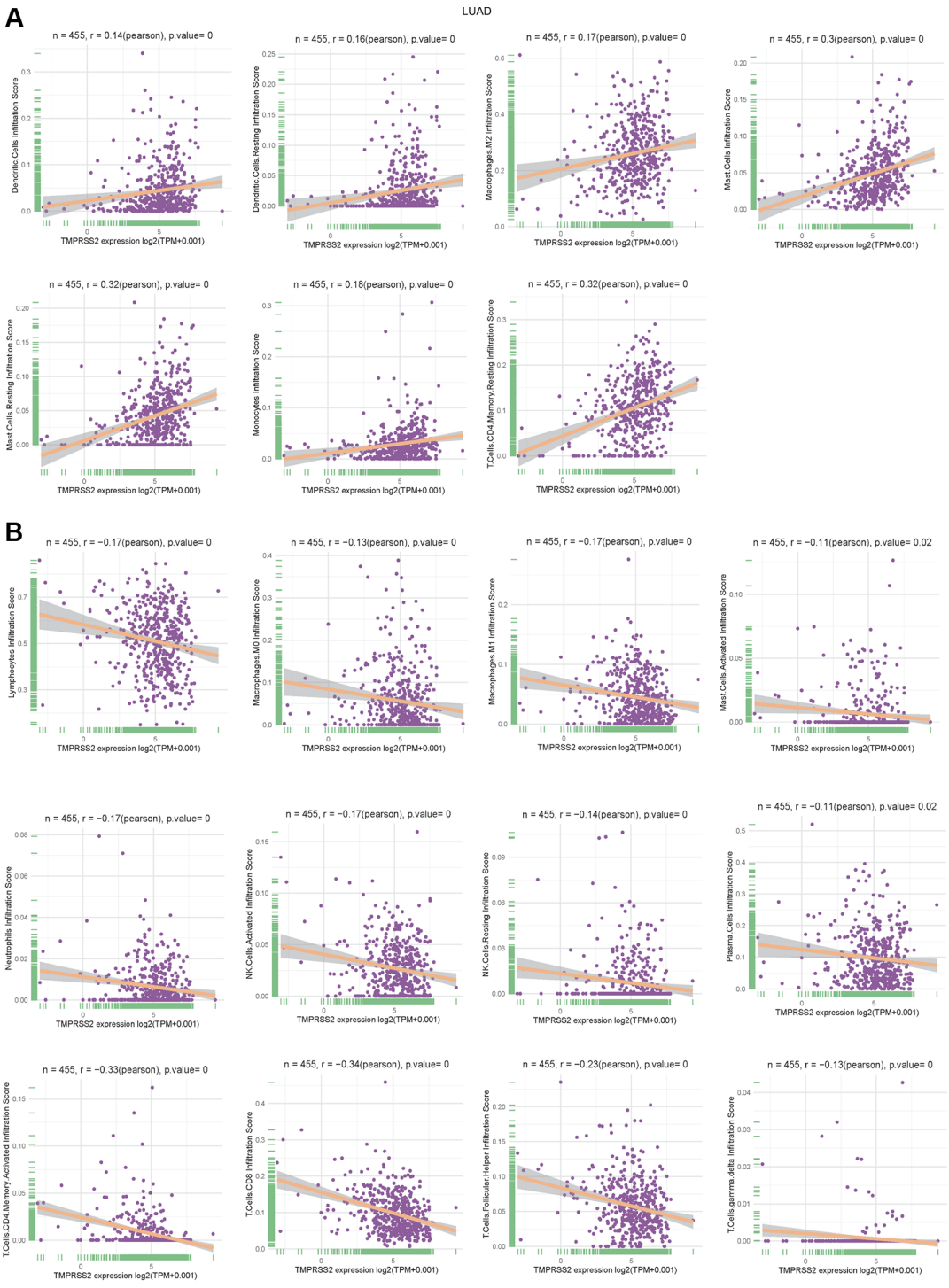
Supplementary Figure 5. Analysis of neighboring gene networks in lung cancer. (**A**) The gene-gene interaction network of TMPRSS2 was constructed using GeneMANIA. (**B**) The PPI network of TMPRSS2 was constructed using STRING. (**C**, **D**) Scatterplots of the correlations between TMPRSS2 expression and SLC45A3 and AR expression in lung cancer using the TIMER and GEPIA databases, respectively. (**E**) Heatmap of correlations between TMPRSS2 expression and other targets of COVID-19 therapy in LUAD and LUSC.



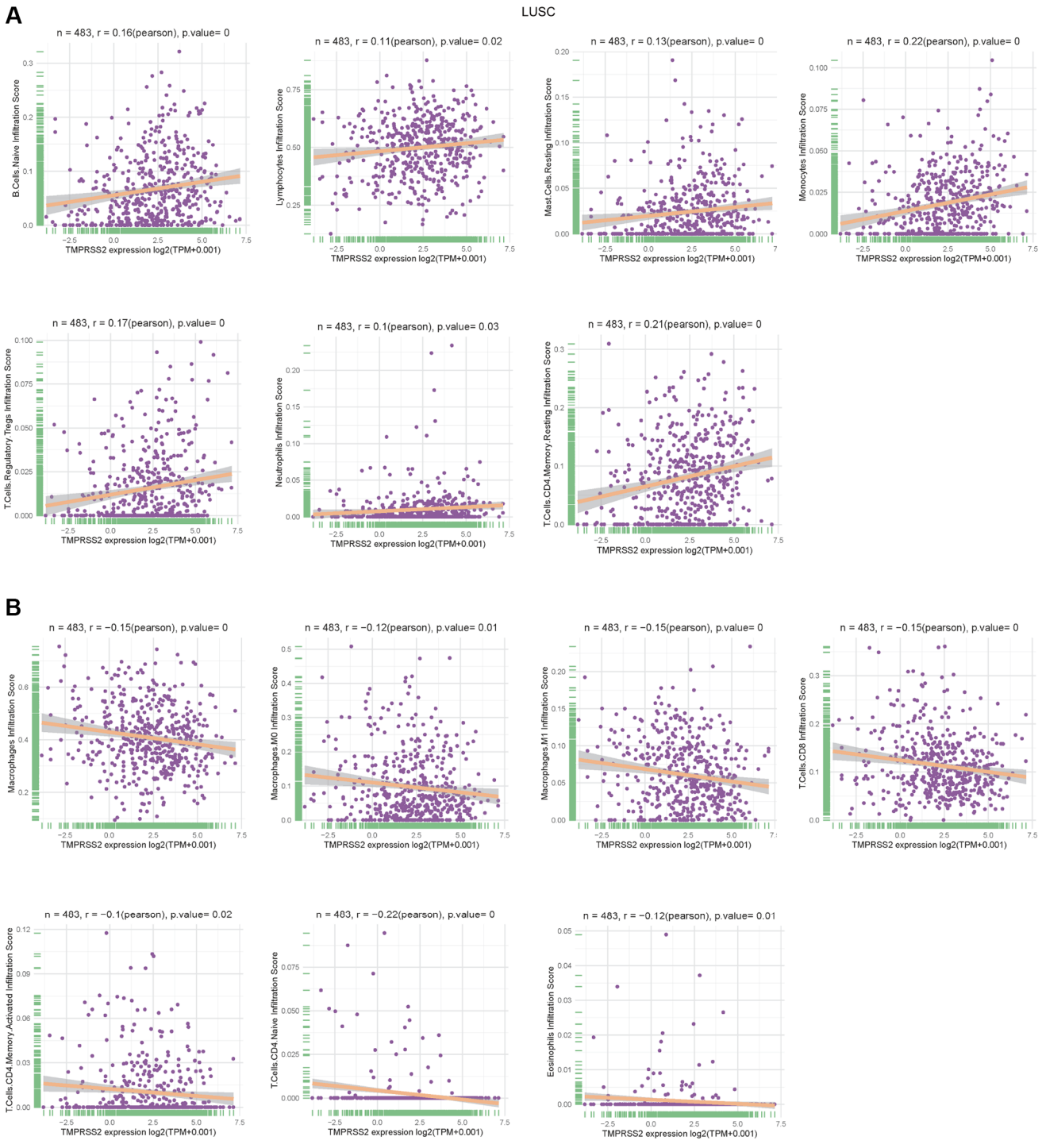
Supplementary Figure 6. GO and KEGG analyses for TMPRSS2 in lung cancer. (**A**, **B**) Heat maps showing the top 50 genes that were negatively associated with TMPRSS2 in LUAD and LUSC, respectively. (**C**, **D**) Top 20 enrichment terms in the MF category in LUAD and LUSC, respectively. (**E**, **F**) Top 20 enrichment terms in the CC category in LUAD and LUSC, respectively.



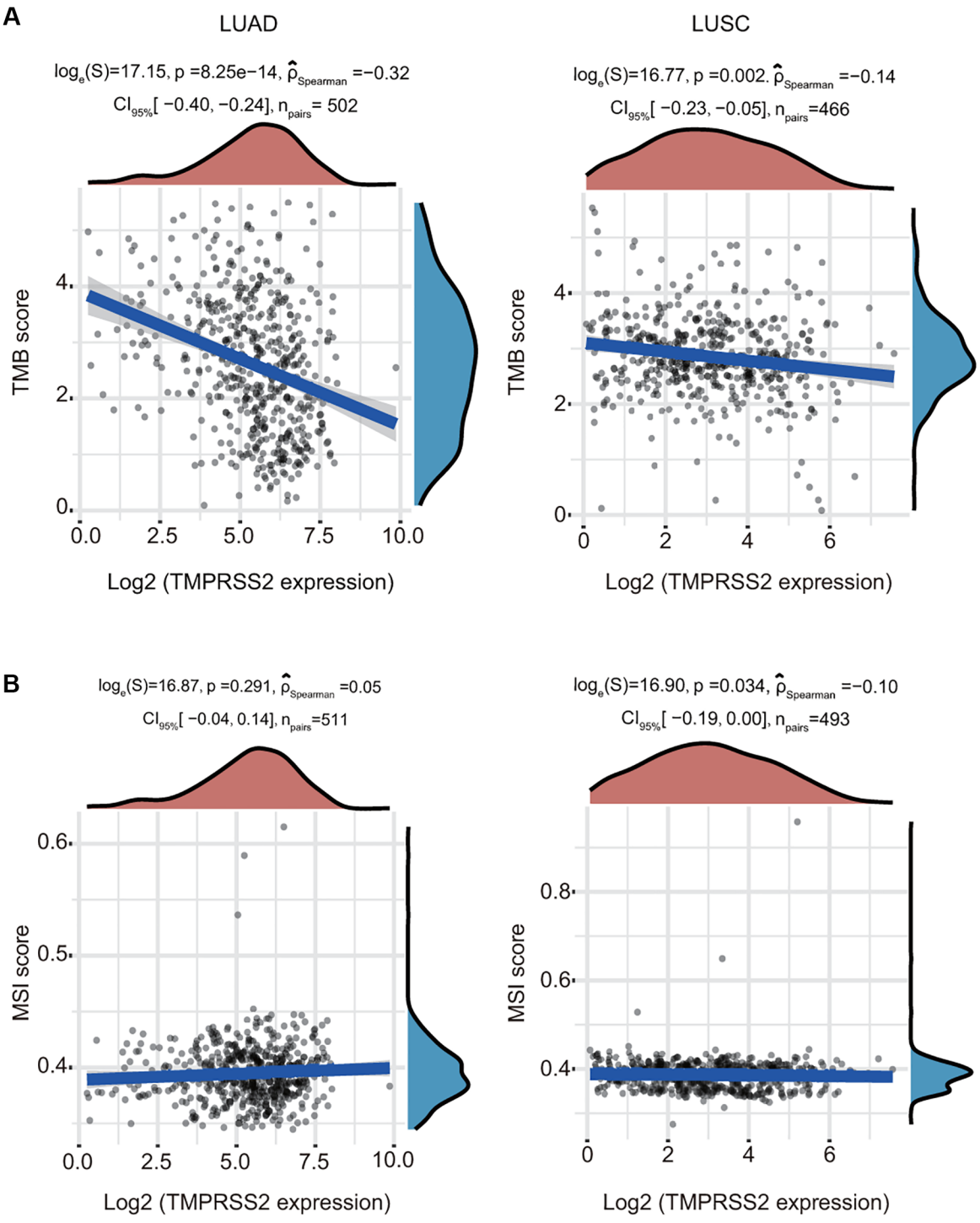
Supplementary Figure 7. (**A**) TMPRSS2 expression was related with various human diseases. (**B**) TMPRSS2 expression was related with multiple cancerous diseases using the Open Targets platform.



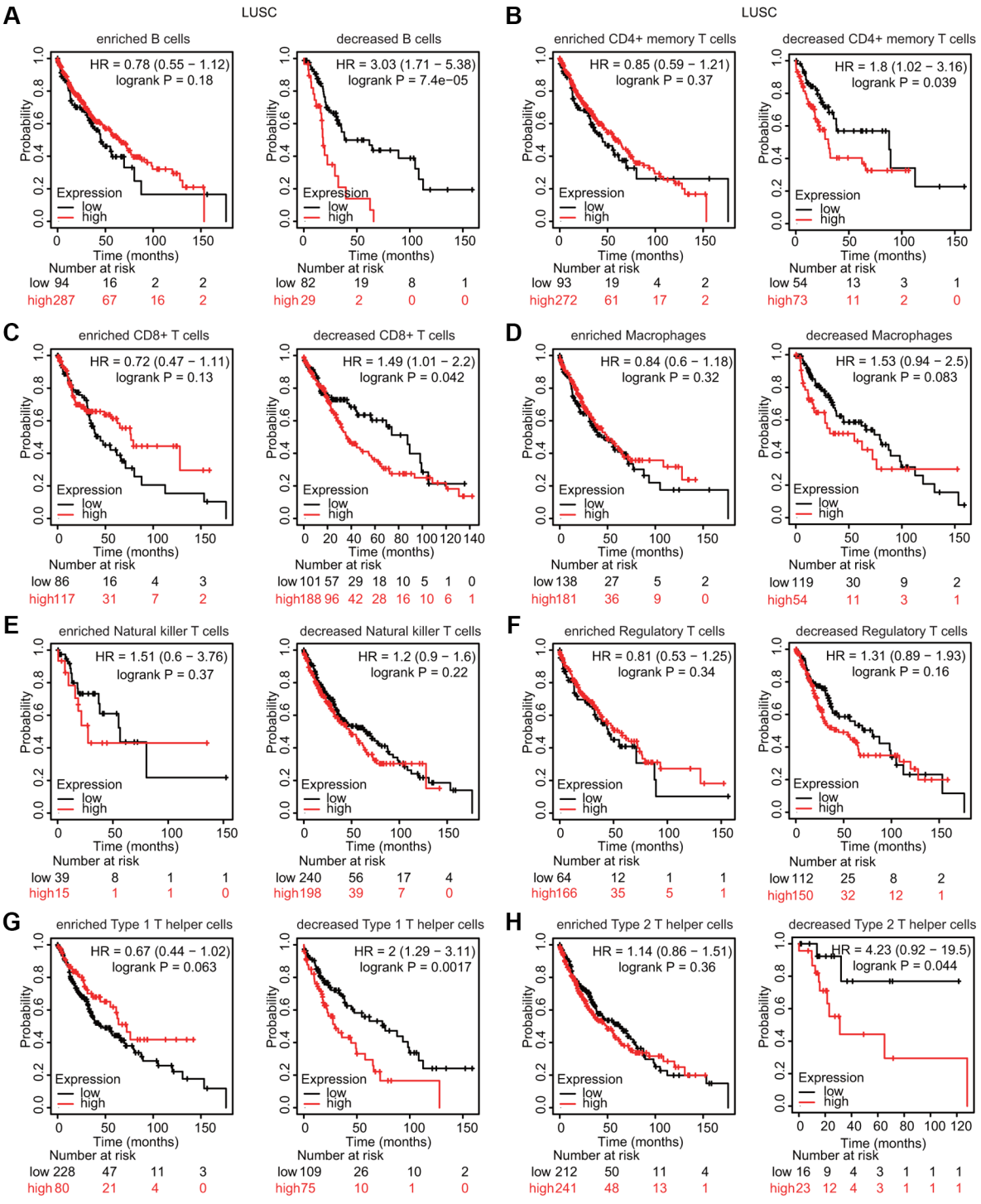
Supplementary Figure 8. (**A**, **B**) TMPRSS2 expression was positively or negatively correlated with the infiltration of different immune cells in LUAD according to the CIBERSORT algorithm.



Supplementary Figure 9. (**A**, **B**) TMPRSS2 expression was positively or negatively correlated with the infiltration of different immune cells in LUSC according to the CIBERSORT algorithm.



Supplementary Figure 10. (**A**, **B**) Correlations between TMPRSS2 expression and TMB and MSI in LUAD and LUSC.



Supplementary Figure 11. (**A**–**H**) Correlations between TMPRSS2 expression and OS in different immune cell subgroups of LUSC patients were examined using the Kaplan-Meier plotter database.

Research Paper

The effect of age on ventilation management and clinical outcomes in critically ill COVID–19 patients—insights from the PRoVENT–COVID study

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ABSTRACT

Introduction: We analyzed the association of age with ventilation practice and outcomes in critically ill COVID–19 patients requiring invasive ventilation.

Methods: Posthoc analysis of the PRoVENT–COVID study, an observational study performed in 22 ICUs in the first 3 months of the national outbreak in the Netherlands. The coprimary endpoint was a set of ventilator parameters, including tidal volume normalized for predicted bodyweight, positive end–expiratory pressure, driving pressure, and respiratory system compliance in the first 4 days of invasive ventilation. Secondary endpoints were other ventilation parameters, the use of rescue therapies, pulmonary and extrapulmonary complications in the first 28 days in the ICU, hospital– and ICU stay, and mortality.

Results: 1122 patients were divided into four groups based on age quartiles. No meaningful differences were found in ventilation parameters and in the use of rescue therapies for refractory hypoxemia in the first 4 days of invasive ventilation. Older patients received more often a tracheostomy, developed more frequently acute kidney injury and myocardial infarction, stayed longer in hospital and ICU, and had a higher mortality.

Conclusions: In this cohort of invasively ventilated critically ill COVID–19 patients, age had no effect on ventilator management. Higher age was associated with more complications, longer length of stay in ICU and hospital and a higher mortality.

INTRODUCTION

The coronavirus disease 2019 (COVID–19) pandemic has resulted in worldwide recurrent surges of patients in need for urgent and intense medical care [1], and as of early–November 2021 5 million patients have died from this new disease [2]. Many hospitalized COVID–19 patients need admission to an intensive care unit (ICU), most often for escalation of respiratory support that includes invasive ventilation [3].

Aging is associated with various changes in lung physiology [4]. Due to changes in the structure of the thoracic cage, aging is known to reduce chest wall compliance. However, lung compliance increases with age because of a decrease in elastic recoil. Second, aging is associated with so–called 'senile emphysema' [5]. Due to a decrease in the supporting structures of lung parenchyma, the risk for early closure of small airways increases which could result in air trapping. The increased incidence of comorbidities in elderly may also mandate a different ventilation approach. For example, the combination of a reduced respiratory system reserve and an increased incidence of pulmonary disease in elderly patients may require a higher FiO_2, while the higher incidence of cardiovascular disease in the elderly may actually reduce the possibility of, for example, ventilation with higher pressures. Indeed, one small prospective cohort study showed that elderly patients with acute respiratory failure received ventilation with lower pressures compared to younger patients [6]. However, this was not confirmed by a more recently published study, showing no age dependent variations in ventilator settings in such patients [7].

Several risk factors for contracting severe COVID–19 have been identified and described. Elderly patients, but also patients with underlying cardiovascular or respiratory conditions are most vulnerable to develop a complicated SARS–CoV–2 infection [8–10], and are at a higher risk for mortality of this disease [11–13]. Aging itself, however, is linked to the development of comorbidities and functional disabilities. Indeed, patients aged > 65 years are three times more often diagnosed with multiple chronic diseases [14], including comorbidities like cancer, cardiovascular diseases, and diabetes mellitus. All these are well–known predictors for mortality [15–17]. Older age is also associated with immunological alterations and inflammation, which may also translate into a higher risk of dying from an infectious disease [16].

It is unknown whether age–related differences exist in ventilator settings in critically ill COVID–19 patients. It also remains uncertain to which extent the association of age with mortality in COVID–19 patients requiring invasive ventilation is mediated by the increased prevalence of comorbidities in elderly patients. In the context of these uncertainties, we assessed the database of a large national observational study [18, 19]. We hypothesized that age has an independent effect on ventilator management and has an association with outcome in critically ill invasively ventilated COVID–19 patients.

MATERIALS AND METHODS

Design, study sites, and participants

This is a posthoc analysis of a national multicenter observational study, named 'Practice of VENTilation in COVID–19 patients' (PRoVENT–COVID) [18]. This study included more than 40% of all critically ill COVID–19 patients admitted to a Dutch ICU in the first 3 months of the national outbreak. The study protocol was approved by the Institutional Review Board of the Amsterdam UMC, location AMC, Amsterdam, the Netherlands on 7 April 2020 (W20_157 # 20.171), and hence at the other 21 hospital that eventually participated in the study. The need for written informed consent was waived because of the observational nature. The study was registered at clinicaltrials.gov (study identifier NCT04346342).

Adult patients were eligible if admitted to the ICU of a participating hospital, and receiving invasive ventilation for respiratory failure related to COVID–19, confirmed by RT–PCR. For the current analysis, we excluded patients that were transferred to an ICU in a non–participating hospital within the first hour of invasive ventilation.

Data collection

Multiple in–person and virtual meetings were organized at the Amsterdam University Medical Centers, location 'AMC', to train data collectors, that were all doctors in training or medical residents. During these meetings, data entry instructions were given, the database structure was explained, and data entry was trained. Each data collector was supervised by an experienced researcher in the domain of critical care. If inaccuracies, outliers and errors were found after data review, queries were sent and resolved by local investigators. Patient characteristics, anthropometric data, medical history, and available severity scores as recorded in the electronic patient records, severity of acute respiratory distress syndrome (ARDS) according to the current Berlin definition for this syndrome [20], and the extent of lung involvement on chest computed tomography or chest radiographs was collected for all patients at

baseline. Different disease severity scores, e.g., the Acute Physiology and Chronic Health Evaluation (APACHE) II or IV score, the Simplified Acute Physiology Score (SAPS) II and the Sequential Organ Failure Assessment (SOFA) score, were used in the participating hospitals. The disease severity score documented in each hospital was collected at baseline, i.e., in the first 24 hours in the ICU. Laboratory test results, hemodynamic parameters, kidney function, fluid balance, and use and dose of continuous sedation, muscle paralysis, and vasopressors were captured daily up to calendar day 4.

Ventilator settings and key ventilation variables and parameters, and the use of adjunctive rescue therapies for refractory hypoxemia, including alveolar recruitment maneuvers, prone positioning, use of neuromuscular blocking agents (NMBAs), and extracorporeal membrane oxygenation (ECMO) was collected at fixed time points 3 times per day (08:00, 16:00 and 24:00) up to calendar day 4 or until death or ICU discharge, if that occurred first. From these three measurement points, the daily mean was calculated for each respiratory variable.

Pulmonary and extrapulmonary events were recorded until ICU day 28, ICU discharge or date of death, whichever came first.

Patients' location and life status were collected up to day 90.

Study endpoints

The coprimary endpoint of this current analysis was a set of 4 key ventilator settings and ventilation parameters: tidal volume normalized for predicted bodyweight ($V_{T\ PBW}$), positive end–expiratory pressure (PEEP), driving pressure (ΔP), and respiratory system compliance (Crs) during the first 4 calendar days.

Secondary endpoints were other ventilation parameters and use of rescue therapies for hypoxemia, pulmonary and extrapulmonary complications, ICU and hospital discharge, the number of days alive and free from invasive ventilation at day 28, and mortality at ICU and hospital discharge and at day 28 and 90.

Definitions

Pulmonary and extrapulmonary events were defined as pneumothorax, tracheostomy, reintubation, acute kidney injury and need for renal replacement therapy, and thromboembolic events, including pulmonary embolism, deep venous thrombosis, ischemic stroke, myocardial infarction, and systemic arterial thrombosis.

V_T per predicted bodyweight (PBW) was calculated as follows:

$$(\text{females})\,\text{PBW}\,(\text{kg}) = 45.5 + 0.91 * (\text{height}\,[\text{cm}] - 152.4) \quad \text{[eq. 1a];}$$

$$(\text{males})\,\text{PWB}\,(\text{kg}) = 50.0 + 0.91 * (\text{height}\,[\text{cm}] - 152.4) \quad \text{[eq. 1b]; and}$$

$$V_{T,\,\text{PBW}}\,(\text{ml/kg}) = V_T\,(\text{ml})/\text{PBW}\,(\text{kg}) \quad \text{[eq. 2].}$$

ΔP and mechanical power (MP) were calculated using the following equations:

$$\Delta\text{P}\,(\text{cm}\,\text{H}_2\text{O}) = \text{peak pressure}\,(\text{Ppeak})\,(\text{cm}\,\text{H}_2\text{O}) - \text{PEEP}\,(\text{cm}\,\text{H}_2\text{O}) \quad \text{[eq. 3]; and}$$

$$\text{MP}\,(\text{J/min}) = 0.098 * V_T\,(\text{liters}) * \text{respiratory rate}\,(\text{RR}) * (\text{Ppeak} - 0.5 * \Delta P) \quad \text{[eq. 4]}$$

Crs was calculated as follows:

$$\text{Crs}\,(\text{ml/cm}\,\text{H}_2\text{O}) = V_T\,(\text{ml}) / \Delta P\,(\text{cm}\,\text{H}_2\text{O}) \quad \text{[eq. 5]}$$

Power calculation

We did not perform a formal power calculation—instead, the number of patients available in the database was used as the sample size.

Statistical analysis plan

Patients were categorized into 4 age groups using the age quartiles. The day of the start of ventilation was merged with the first full calendar and named 'day 1'. The following days were named 'day 2' and 'day 3'. No assumptions for missing data were made.

Categorical variables are presented as numbers and proportions, continuous variables are reported with median and interquartile ranges. Age groups were compared using the Kruskal–Wallis test for continuous variables and Fisher exact tests for categorical variables. If differences were found, a posthoc Dunn test was used for pairwise comparison.

Distribution plots were constructed to show the key ventilator parameters for the four age groups. Time-to-event outcomes are presented in Kaplan–Meier curves, and age groups are compared with the Log–rank test.

To adjust for the unequal distribution of effect modifiers between the 4 age groups, multivariable models were

made for ICU and hospital mortality, and 28– and 90-day mortality. The following variables were considered for adjustment in these models: (i.) gender; (ii.) body mass index (BMI); (iii.) history of hypertension, heart failure, diabetes mellitus, chronic kidney disease, chronic obstructive pulmonary disease, active hematological or solid cancer; (iv.) use of angiotensin-converting enzyme inhibitors, use of angiotensin II receptor blockers, and use of vasopressor or inotropic medication; (v.) PaO_2 to FiO_2 ratio; and (vi.) mean arterial blood pressure, heart rate, plasma creatinine, fluid balance, and arterial pH. These baseline covariates were selected according to clinical relevance and as used in previous studies [18, 21].

All analyses were conducted in R, version 4.0.5. A $P < 0.05$ was considered statistically significant.

RESULTS

Participants

Patient flow is shown in Supplementary Figure 1. A total of 1340 patients in 22 ICUs were screened for eligibility; major reasons for exclusions were that patients had an alternate diagnosis or did not receive invasive ventilation. Of the remaining 1122 patients, the median age was 65 [57 to 72] years. Baseline demographics of the 4 age groups are presented in Table 1. Older patients were shorter, weighed less, had a lower BMI and were more often diagnosed with a medical history of arterial hypertension, heart failure, diabetes mellitus, or COPD. Home medication like angiotensin–converting enzyme inhibitors and blockers, beta–blockers, statins, and calcium channel blockers were more often used at home in the higher age groups. At the first day of invasive ventilation, older patients were more often in need of vasopressors and inotropic drugs, and older patients had a higher cumulative fluid balance and a lower urine output.

Ventilation characteristics

Key ventilator settings are shown in Table 2, Figure 1, and Supplementary Figures 2–5. On the first day of ventilation, median $V_{T\ PBW}$, PEEP, ΔP and Crs were largely similar between the 4 age groups. Some differences reached statistical significance, but differences were too small to have a clinical meaning.

Mechanical power and peak pressure decreased from the younger to the older age groups at the first day of ventilation (Table 2). The difference in mechanical power and peak pressure disappeared in subsequent days (Supplementary Table 1). $EtCO_2$ was lower but $PaCO_2$ was higher in older age groups, and PaO_2 was lower in the second age quartile (Table 2); only the difference in $EtCO_2$ persisted in subsequent days (Supplementary Table 1)

Use of adjunctive therapies for refractory hypoxemia was not affected by age, except for the use of NMBAs, which was less used with higher age (Table 3).

Pulmonary and extrapulmonary events

Pulmonary and extrapulmonary complications are presented in Table 3 and Supplementary Table 2. Tracheostomy was more often used in the older compared to the youngest patients. No differences in other pulmonary events were found. There was no effect of age on thrombotic complications, only the incidence of myocardial infarction was higher in the older age groups compared to the younger age groups. Acute kidney injury (AKI) occurred less often in the youngest age group compared to the older age groups, as was the need for renal replacement therapy.

Outcomes

Patient outcomes are shown in Table 3, Supplementary Table 2 and Figure 2. In survivors, length of hospital and ICU stay increased while number of ventilator–free days decreased from the younger to the older age groups. Mortality rates increased from the lowest to the higher age group. After adjustment from effect modifiers, ICU– and hospital mortality, and 28– and 90–day were all higher in older patients (Supplementary Tables 3, 4).

DISCUSSION

The results of this posthoc analysis of the PRoVENT–COVID study can be summarized as follows: (i.) there were no clinically meaningful differences in the key ventilator parameters between the 4 age groups; (ii.) on the first calendar day, mechanical power and peak pressure were lower in older patients but this effect disappeared in the succeeding days; (iii.) on the first four calendar days, $EtCO_2$ was lower while $PaCO_2$ was slightly higher in older patients; (iv) use of NMBAs was lower in older patients; (v) tracheostomy was more often used in older patients; (vi.) the incidence of AKI and the need for renal replacement therapy, and myocardial infarction was higher in older patients; (vii.) older patients stayed longer in the ICU and hospital; and (viii.) had higher mortality rates.

Our study has several strengths. The study included a large number of centers, both academic and non–academic, increasing the generalizability of the findings. Data were collected in a short time interval of

Table 1. Patient characteristics according to age category at baseline.

	Age 22 to 57 years (*n* = 287)	Age 58 to 65 years (*n* = 286)	Age 66 to 72 years (*n* = 283)	Age 73 to 85 years (*n* = 266)	*P* value
Age, years	52.0 [47.0 to 55.0]	62.0 [60.0 to 64.0]	69.0 [67.0 to 71.0]	75.0 [74.0 to 77.0]	<0.001
Male	200 (69.7)	217 (75.9)	203 (71.7)	197 (74.1)	0.370
Height, cm	178.0 [170.0 to 185.0]	178 [170.0 to 184.0]	175.0 [170.0 to 180.0]	174.0 [168.5 to 180.0]	<0.001
Weight, kg	90.0 [80.8 to 105.0]	89.0 [78.2 to 98.0]	85.0 [75.6 to 92.2]	82.0 [75.0 to 90.0]	<0.001
Body Mass Index, kg/m^2	28.9 [26.2 to 32.7]	27.7 [25.4 to 30.6]	27.2 [24.8 to 29.7]	27.0 [24.9 to 29.4]	<0.001
Severity of illness*					
SAPS II, % (no)	35.7 (99/277)	34.3 (92/268)	33.6 (91/271)	30.8 (77/250)	
*Modified SAPS II	24.0 [19.0 to 29.0]	24.0 [19.0 to 31.0]	24.5 [19.0 to 32.0]	26.0 [20.0 to 34.0]	0.361
APACHE II, no (%)	26.0 (72/277)	25.4 (68/268)	17.7 (48/271)	22.4 (56/250)	
*Modified APACHE II	12.0 [10.0 to 15.0]	12.0 [9.0 to 15.0]	15.0 [9.0 to 19.0]	15.0 [10.0 to 20.0]	0.026
APACHE IV, no (%)	45.5 (126/277)	40.7 (109/268)	41.7 (113/271)	36.8 (92/250)	
*Modified APACHE IV	44.0 [37.2 to 55.0]	44.0 [35.0 to 56.5]	49.0 [36.8 to 59.2]	49.0 [34.8 to 62.0]	0.469
SOFA, no (%)	53.4 (148/227)	54.1 (145/268)	46.5 (126/271)	44.4 (111/250)	
SOFA	7.0 [5.0 to 8.0]	7.0 [6.0 to 10.0]	7.0 [6.0 to 10.0]	8.0 [7.0 to 12.5]	<0.001
Comorbidities					
Arterial hypertension	53 (18.5)	105 (36.7)	108 (38.2)	114 (42.9)	<0.001
Heart failure	3 (1.0)	10 (3.5)	16 (5.7)	20 (7.5)	<0.001
Diabetes mellitus	44 (15.3)	62 (21.7)	80 (28.3)	64 (24.1)	0.002
Chronic kidney disease	8 (2.8)	14 (4.9)	9 (3.2)	16 (6.0)	0.204
Baseline creatinine	71.0 [60.0 to 87.0]	77.0 [64.0 to 98.0]	78.0 [63.0 to 98.0]	84.0 [66.8 to 111.2]	<0.001
Liver cirrhosis	2 (0.7)	0 (0.0)	0 (0.0)	1 (0.4)	0.329
Chronic obstructive pulmonary disease	8 (2.8)	25 (8.7)	34 (12.0)	21 (7.9)	<0.001
Active hematological neoplasia	3 (1.0)	5 (1.7)	4 (1.4)	4 (1.5)	0.911
Active solid neoplasia	3 (1.0)	7 (2.4)	8 (2.8)	10 (3.8)	0.193
Neuromuscular disease	4 (1.4)	0 (0.0)	2 (0.7)	2 (0.8)	0.258
Immunosuppression	7 (2.4)	8 (2.8)	5 (1.8)	4 (1.5)	0.710
Previous medication					
Systemic steroids	6 (2.1)	8 (2.8)	10 (3.5)	14 (5.3)	0.216
Inhalation steroids	34 (11.8)	37 (12.9)	33 (11.7)	21 (7.9)	0.244
Angiotensin-converting enzyme inhibitor	25 (8.7)	45 (15.7)	62 (21.9)	57 (21.4)	<0.001
Angiotensin II receptor blocker	18 (6.3)	35 (12.2)	30 (10.6)	44 (16.5)	0.002
Beta-blockers	28 (9.8)	52 (18.2)	63 (22.3)	68 (25.6)	<0.001
Insulin	16 (5.6)	22 (7.7)	21 (7.4)	19 (7.1)	0.744
Metformin	29 (10.1)	47 (16.4)	52 (18.4)	47 (17.7)	0.020
Statins	35 (12.2)	76 (26.6)	110 (38.9)	109 (41.0)	<0.001
Calcium channel blockers	29 (10.1)	45 (15.7)	59 (20.8)	64 (24.1)	<0.001
Transferred under invasive ventilation from another hospital	59 (20.6)	53 (18.5)	48 (17.0)	41 (15.4)	0.436
Days between admission and start of invasive ventilation	0.0 [0.0 to 0.0]	0.0 [0.0 to 0.0]	0.0 [0.0 to 0.0]	0.0 [0.0 to 0.0]	0.508
Use of non-invasive mechanical ventilation before intubation	28/259 (10.8)	14/256 (5.5)	24/258 (9.3)	19/236 (8.1)	0.152
Duration of non-invasive ventilation, hours	7.0 [2.0 to 23.0]	7.0 [3.5 to 19.0]	8.0 [2.8 to 9.5]	8.0 [1.0 to 17.0]	1.000
Chest CT-scan performed at baseline	111/276 (40.2)	93/270 (34.4)	78/269 (29.0)	81/257 (31.5)	0.023
Percentage lung parenchyma affected					0.561
0%	7/111 (6.3)	3/93 (3.2)	3/78 (3.8)	1/81 (1.2)	
25%	29/111 (26.1)	27/93 (29.0)	29/78 (37.2)	31/81 (38.3)	
50%	38/111 (34.2)	26/93 (28.0)	21/78 (26.9)	22/81 (27.2)	
75%	30/111 (27.0)	33/93 (35.5)	19/78 (24.4)	22/81 (27.2)	

100%	7/111 (6.3)	4/93 (4.3)	6/78 (7.7)	5/81 (6.2)	
Chest x-ray performed at baseline	136/162 (84.0)	152/176 (86.4)	157/185 (84.9)	157/176 (89.2)	0.506
Quadrants affected					0.810
1	13 (9.8)	12 (7.8)	8 (5.0)	9 (5.8)	
2	32 (24.1)	37 (24.0)	38 (23.8)	32 (20.8)	
3	34 (25.6)	39 (25.3)	45 (28.1)	50 (32.5)	
4	54 (40.6)	66 (42.9)	69 (43.1)	63 (40.9)	
Laboratory tests					
pH	7.4 [7.3 to 7.4]	7.4 [7.3 to 7.4]	7.4 [7.3 to 7.4]	7.3 [7.3 to 7.4]	<0.001
PaO_2	10.7 [9.2 to 14.2]	10.3 [8.8 to 12.6]	10.9 [9.5 to 13.3]	11.2 [9.7 to 13.3]	0.008
SaO_2	95.0 [93.0 to 97.4]	94.2 [92.0 to 96.8]	95.0 [93.0 to 97.0]	95.0 [93.0 to 97.0]	0.030
$PaCO_2$	5.6 [4.9 to 6.5]	5.9 [5.0 to 6.9]	6.1 [5.3 to 7.1]	5.9 [5.0 to 6.9]	0.003
Lactate	1.1 [0.9 to 1.4]	1.1 [0.9 to 1.4]	1.2 [0.9 to 1.5]	1.2 [1.0 to 1.6]	0.002
Worst PaO_2/FiO_2 ratio, mm Hg	126.6 [94.7 to 164.5]	117.9 [91.8 to 160.3]	120.2 [96.1 to 157.3]	126.2 [97.4 to 161.6]	0.401
Need for advanced support					
Continuous sedation	277/287 (96.5)	276/286 (96.5)	267/277 (95.0)	253/263 (95.1)	0.691
Need for vasopressor use	198/287 (69.0)	223/286 (78.0)	225/281 (80.1)	217/266 (81.6)	0.002
Need for inotropic use	6/287 (2.1)	6/286 (2.1)	16/281 (5.7)	17/266 (6.4)	0.009
Fluid balance, mL	418.0 [-126.0 to 1206.0]	513.0 [-26.3 to 1209.0]	456.1 [-25.5 to 1252.8]	780.0 [144.0 to 1557.0]	0.001
Urine output, mL	875.0 [511.2 to 1377.5]	657.0 [350.0 to 1120.0]	720.0 [370.0 to 1165.0]	505.0 [255.0 to 877.5]	<0.001

Data presented as median with interquartile range [25th to 75th quartile] or n (%). *Age component is removed from the APACHE and SAPS Score. *Total numbers are different because different scores were used in the participating hospitals.
SAPS, Simplified Acute Physiology Score; APACHE, Acute Physiology and Chronic Health Evaluation; SOFA, Sequential Organ Failure Assessment; CT, Computed Tomography.

Table 2. Characteristics of mechanical ventilation and laboratory results in the first day of ventilation.

	Age 22 to 57 years (*n* = 287)	**Age 58 to 65 years (*n* = 286)**	**Age 66 to 72 years (*n* = 283)**	**Age 73 to 85 years (*n* = 266)**	***P* value**
Mode of mechanical ventilation					
Volume control	32/271 (11.8)	35/267 (13.1)	33/267 (12.4)	41/248 (16.5)	0.398
Pressure control	163/271 (60.1)	153/267 (57.3)	149/267 (55.8)	123/248 (49.6)	0.103
Pressure support	12/271 (4.4)	20/267 (7.5)	13/267 (4.9)	12/248 (4.8)	0.380
Synchronized Intermitted Mandatory Ventilation	19/271 (7.0)	12/267 (4.5)	25/267 (9.4)	22/248 (8.9)	0.131
Airway Pressure Release Ventilation	9/271 (3.3)	10/267 (3.7)	10/267 (3.7)	5/248 (2.0)	0.652
INTELLIVENT-Adaptive Support Ventilation	11/271 (4.1)	10/267 (3.7)	12/267 (4.5)	11/248 (4.4)	0.971
Other	25/271 (9.2)	27/267 (10.1)	25/267 (9.4)	34/248 (13.7)	0.310
Use of assisted ventilation	76/287 (26.5)	78/282 (27.7)	88/283 (31.1)	88/265 (33.2)	0.285
Tidal volume (n/N), mL/kg PBW*	(274/287) 6.4 [5.8 to 7.0]	(274/286) 6.4 [5.9 to 7.1]	(263/283) 6.5 [5.9 to 7.1]	(243/266) 6.5 [6.0 to 7.1]	0.445
PEEP, (n/N) cmH2O*	(287/287) 13.0 [11.0 to 15.0]	(286/286) 12.7 [11.0 to 14.6]	(279/283) 13.0 [10.7 to 14.8]	(262/266) 12.2 [10.8 to 14.2]	0.314
Driving pressure (n/N), cmH2O*	(264/287) 14.7 [12.5 to 17.0]	(265/286) 13.8 [11.7 to 16.3]	(252/283) 13.2 [11.3 to 15.7]	(227/266) 13.5 [11.6 to 15.7]	<0.001
Compliance (n/N), mL/cmH2O*	(256/287) 32.4 [25.9 to 38.3]	(258/286) 33.8 [27.1 to 41.7]	(241/283) 34.7 [27.7 to 43.3]	(215/266) 32.6 [27.3 to 40.7]	0.073
Mechanical power (n/N), J/min*	(256/287) 19.2 [16.0 to 23.7]	(257/286) 19.3 [15.9 to 23.1]	(241/283) 17.9 [14.7 to 22.3]	(214/266) 17.2 [14.6 to 20.9]	<0.001
Peak pressure (n/N), cmH_2O*	(264/287) 27.7 [25.0 to 30.8]	(267/286) 26.7 [23.3 to 30.0]	(257/283) 26.0 [23.3 to 29.2]	(227/266) 26.2 [23.6 to 29.0]	<0.001
Total respiratory rate (n/N), breaths per minute*	(287/287) 22.0 [20.0 to 24.3]	(286/286) 22.0 [19.5 to 24.5]	(282/283) 21.3 [19.3 to 24.0]	(258/266) 21.3 [19.1 to 23.7]	0.053
Minute ventilation (n/N), L/min*	(275/287) 9.8 [8.6 to 11.4]	(277/286) 10.0 [8.5 to 11.6]	(269/283) 9.6 [8.2 to 11.3]	(245/266) 9.3 [8.2 to 10.6]	0.005

Minute volume corrected (n/N), mL/kg/min PBW*	(274/287) 139.1 [121.9 to 158.3]	(274/286) 139.9 [124.8 to 162.9]	(263/283) 137.7 [123.7 to 159.6]	(243/266) 137.2 [122.8 to 155.0]	0.782
FiO_2 (n/N)*	(286/287) 0.6 [0.5 to 0.7]	(286/286) 0.6 [0.5 to 0.7]	(281/283) 0.6 [0.5 to 0.7]	(258/266) 0.6 [0.5 to 0.7]	0.283
PaO_2 (n/N), mmHg*	(284/287) 81.0 [71.5 to 99.3]	(286/286) 78.7 [71.3 to 93.4]	(280/283) 82.4 [72.7 to 95.4]	(264/266) 83.3 [75.0 to 96.0]	0.018
$PaCO_2$ (n/N), mmHg*	(284/287) 42.9 [38.3 to 48.4]	(286/286) 44.6 [39.8 to 49.5]	(280/283) 46.1 [39.9 to 52.0]	(264/266) 45.0 [39.1 to 50.9]	0.002
$EtCO_2$ (n/N), mmHg*	(264/287) 38.0 [33.8 to 43.8]	(257/286) 37.7 [33.3 to 42.8]	(261/283) 36.3 [31.9 to 42.0]	(231/266) 35.3 [31.6 to 39.9]	<0.001

Data presented as median with interquartile range [25th to 75th quartile] or n (%). *Mean of all values available at the first day of ventilation. Total numbers are different because of missing or unmeasured values. $EtCO_2$, End-Tidal Carbon Dioxide; FiO_2, inspired fraction of oxygen; ICU, Intensive Care.

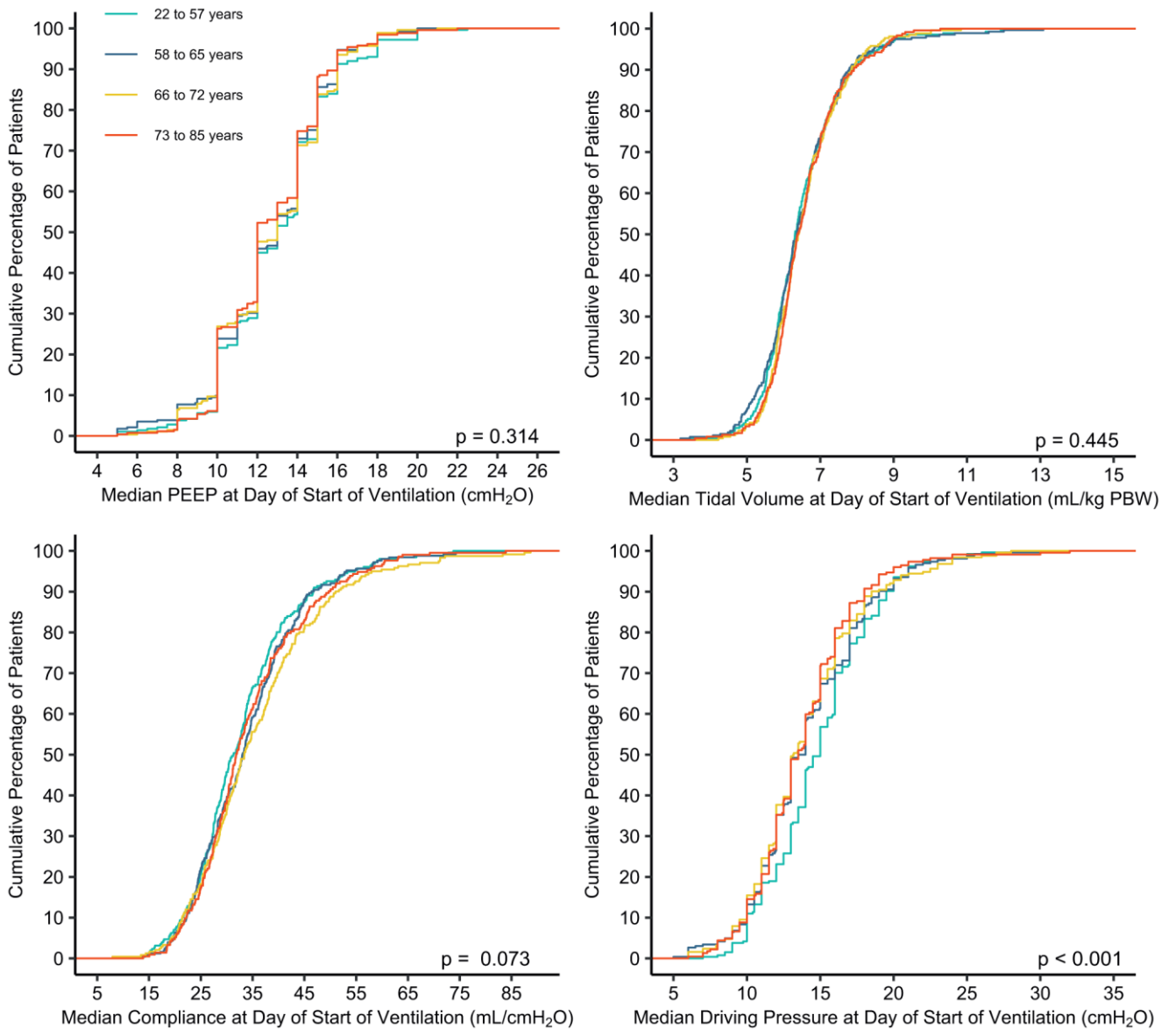

Figure 1. Cumulative frequency distribution of median PEEP, tidal volume, compliance and driving pressure at start day of invasive ventilation. Mean values were calculated from three or four measurements available on the first day of ventilation. The Kruskal-Wallis test was used to calculate p-values.

Table 3. Clinical outcome according to age group.

	Age 22 to 57 years (*n* = 287)	Age 58 to 65 years (*n* = 286)	Age 66 to 72 years (*n* = 283)	Age 73 to 85 years (*n* = 266)	*P* value
28-day mortality	36/281 (12.8)	59/279 (21.1)	100/279 (35.8)	123/263 (46.8)	<0.001
90-day mortality	46/255 (18.0)	72/251 (28.7)	120/267 (44.9)	145/242 (59.9)	<0.001
In hospital mortality	43/259 (16.6)	71/256 (27.7)	113/255 (44.3)	140/252 (55.6)	<0.001
ICU mortality	42/277 (15.2)	71/278 (25.5)	110/274 (40.1)	133/262 (50.8)	<0.001
Length of hospital stay, days	24.0 [17.0 to 33.0]	26.0 [16.0 to 41.0]	22.0 [14.0 to 39.0]	21.5 [10.0 to 36.0]	0.008
Length of hospital stay in survivors, days	25.0 [18.5 to 35.5]	30.0 [20.0 to 46.5]	32.5 [20.3 to 49.8]	33.0 [25.8 to 52.0]	<0.001
Length of ICU stay, days	15.0 [10.0 to 23.0]	17.0 [10.0 to 30.0]	16.0 [8.3 to 26.0]	14.0 [7.0 to 25.0]	0.037
Length of ICU stay in survivors, days	15.0 [10.0 to 22.8]	20.0 [12.0 to 31.0]	18.0 [10.0 to 34.0]	20.0 [13.0 to 38.0]	<0.001
Ventilator-free days at day 28	13.0 [0.0 to 19.0]	4.0 [0.0 to 17.0]	0.0 [0.0 to 14.2]	0.0 [0.0 to 9.7]	<0.001
Duration of ventilation, days	13.0 [9.0 to 21.0]	15.0 [9.0 to 26.0]	15.0 [8.0 to 24.0]	13.0 [6.0 to 22.0]	0.023
Duration of ventilation in survivors, days*	13.0 [8.0 to 21.2]	17.0 [10.0 to 28.3]	17.0 [10.0 to 31.0]	19.0 [12.0 to 34.0]	<0.001
Tracheostomy*	35/283 (12.4)	62/284 (21.8)	48/280 (17.1)	45/265 (17.0)	0.029
Reintubation*	32/282 (11.3)	42/284 (14.8)	33/278 (11.9)	33/264 (12.5)	0.631
Pneumothorax*	2/283 (0.7)	3/275 (1.1)	2/267 (0.7)	2/259 (0.8)	0.970
Thrombotic complications*&	72/287 (25.1)	95/286 (33.2)	74/283 (26.1)	78/266 (29.3)	0.135
Pulmonary embolism	55/287 (19.2)	75/286 (26.2)	61/283 (21.6)	58/266 (21.8)	0.236
Deep vein thrombosis	17/287 (5.9)	20/286 (7.0)	9/283 (3.2)	11/266 (4.1)	0.156
Ischemic stroke	3/287 (1.0)	10/286 (3.5)	8/283 (2.8)	10/266 (3.8)	0.148
Myocardial infarction	2/287 (0.7)	0/286 (0.0)	7/283 (2.5)	7/266 (2.6)	0.007
Systemic arterial thrombosis	1/287 (0.3)	1/286 (0.3)	2/283 (0.7)	0/266 (0.0)	0.805
Acute kidney injury*	89/287 (31.0)	140/285 (49.1)	126/281 (44.8)	141/265 (53.2)	<0.001
Need for renal replacement*	35/287 (12.2)	62/286 (21.7)	57/283 (20.1)	51/266 (19.2)	0.013
Adjunctive therapies refractory hypoxemia**	162/284 (57.0)	174/282 (61.7)	159/282 (56.4)	152/265 (57.4)	0.563
Prone positioning	156/284 (54.9)	169/282 (59.9)	155/282 (55.0)	145/265 (54.7)	0.533
Alveolar recruitment maneuver	15/242 (6.2)	16/239 (6.7)	18/239 (7.5)	15/214 (7.0)	0.946
Other adjunctive therapies**	156/287 (54.4)	134/286 (46.9)	143/283 (50.5)	104/266 (39.1)	0.003
Neuromuscular blocking agents	156/287 (54.4)	133/286 (46.5)	141/283 (49.8)	104/266 (39.1)	0.003
Extracorporeal membrane oxygenation	7/285 (2.5)	2/282 (0.7)	2/278 (0.7)	1/262 (0.4)	0.142

Data presented as median with interquartile range [25th to 75th quartile] or n (%). Totals are different due to missing data. *Assessed at day 28. **Assessed in the first four days of ventilation. &One could have more than one thrombotic complication. Total numbers are different because of missing or unmeasured values.
ICU, Intensive Care Unit.

3 months, which minimizes the risk of changes in care over time. Data were collected by trained data collectors, which improved the quality of the data. Patients were followed until day 90, enabling for reporting on outcomes after stay in ICU. Of note, median age and other baseline characteristics are comparable to that in other studies [22, 23]. Also, in line with previous studies, the second and third age group had an evidently smaller range than the first and last age group, suggesting that middle–aged patients were the most prominent group admitted to the ICU.

Our findings suggest that ventilator management is not affected by age. Indeed, we found only minor, clinical meaningless, differences in key ventilator variables. The younger age groups had a higher BMI that could, at least

in part, explain the higher median ΔP and Ppeak, and the higher mechanical power. Indeed, with a higher BMI higher thoracic pressures may be needed due to an increased stiffness of the chest wall [24]. Previous studies have shown higher $EtCO_2$ values in older patients [25–27], but this was not seen in our cohort. Actually, the opposite relation between $EtCO_2$ and age could be explained by the higher BMI in the younger age group, as an higher BMI may be associated with an increased production of carbon dioxide [28]. Of note, on the first day of mechanical ventilation, we did find a slightly higher $PaCO_2$ but lower $EtCO_2$ in older patients than in younger patients, but this difference disappeared in the following days. The age dependent reduction in body mass could also explain the lower use of NMBAs in older patients [29]. An association of higher age with lower use of NMBAs has been described before [30]. Other explanations for these differences include age–related differences in clearance of NMBAs, and maybe also the higher incidence of acute kidney injury (AKI) in older patients [29]. As AKI also affects clearance of opioids [31], the higher effective dose of opioids may have prevented use of NMBAs as well. Furthermore, physicians might be reluctance to use NMBAs in elderly patients because of the increased risk of prolonged immobility and thus ICU–acquired weakness [32].

Age is known to be a risk factor for complications like AKI, need for renal replacement therapy, and myocardial infarction [33–36]. Therefore, the increased incidence of these complications in older age groups was expected.

We found a strong association of age with mortality. This is, at least in part, in line with previous studies showing that age is a risk factor for mortality in invasively ventilated ICU patients in general [37–40], and in COVID–19 in particular [13, 41–43]. After adjusting for comorbidities and other effect modifiers, mortality rates remained significantly higher in the older patients. The 28–day mortality rate in our oldest age group was higher than that reported in a prospective study performed in elderly COVID–19 patients [44]. Interestingly, in that study it was shown that when patients were classified according to their frailty scale, mortality increased in vulnerable and frail patients. The level of frailty defines how vulnerable patients are for both physical and psychosocial factors. Frailty can be considered as a marker of biological age and, in addition to calendar age, can provide important prognostic information about clinical outcomes of ICU patients [44, 45]. Unfortunately, frailty was not, or incomplete reported in the medical records in the

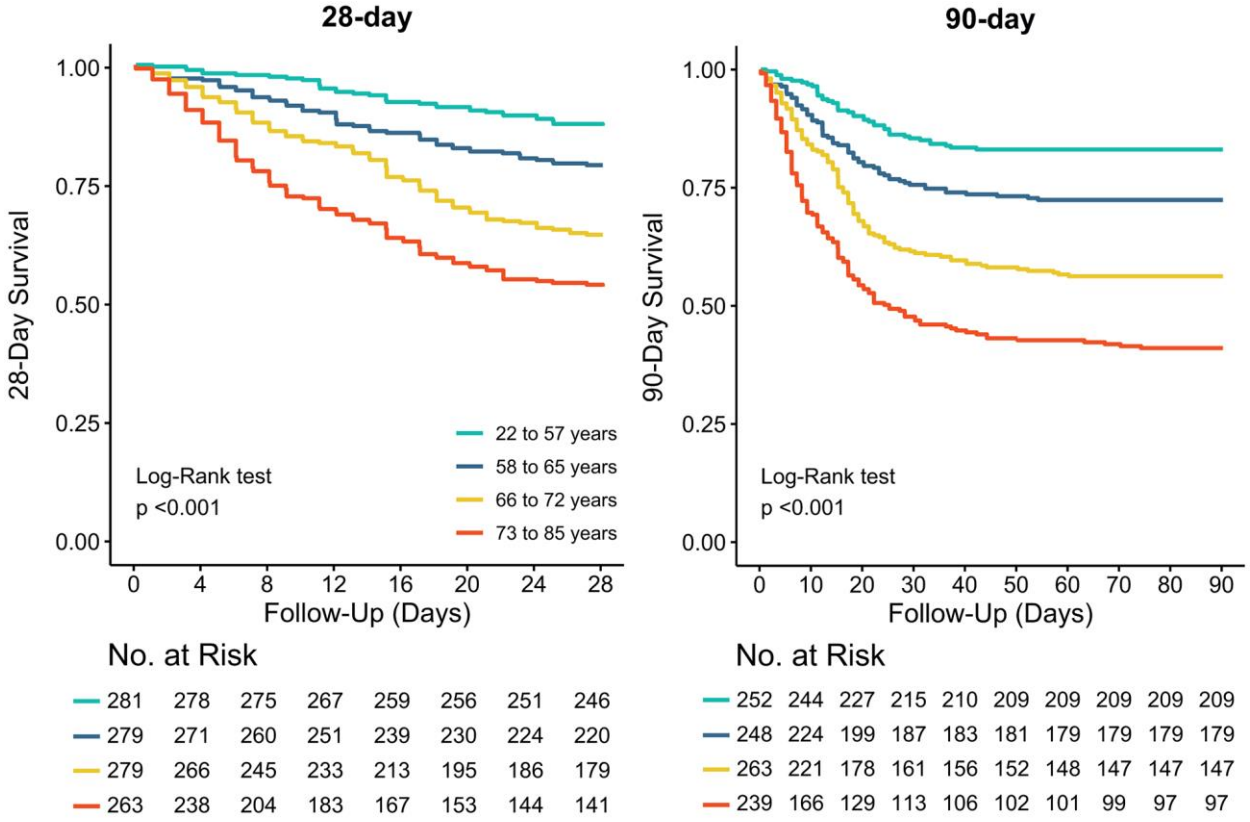

Figure 2. Kaplan-Meier curves for 28-day and 90-day mortality per age group. The Log-Rank test was used to calculate P values.

hospitals that participated in our study, but taken together the differences in mortality between our study and the previous study [44] suggest that patients in our cohort could have been frail more often.

In survivors, older patients stayed longer in the ICU and in the hospital, had a higher incidence of tracheostomy, and received ventilation for more days than younger patients. This may suggest that treatment discontinuation was not more common in elderly patients, but this could also be explained by the fact that older patients may have had already further disease progression or were in a higher need for supportive care. As data on treatment discontinuation were not collected in this analysis, this remains uncertain.

The findings of our study expand the current knowledge about the effects of age on ventilator management and outcomes in critically ill invasively ventilated COVID–19 patients. Lung–protective ventilation was well applied during the first COVID–19 outbreak, also in older patients. The higher mortality rates in older patients could help in decision–making about preventive measures. For example, these findings support guidelines to prioritize the elderly in vaccination programs. These insights may also further support a patient in deciding whether, and to what extent, ICU admission is still desirable.

Our analysis has several limitations. First, the question arises whether 'door selection' for ICU admission may has occurred. Particularly in the elderly, there is a possibility that ICU admission may no longer be considered beneficial if there is a relatively severe disease or premorbid functioning. Unfortunately, we could not collect data on 'Do Not Resuscitate' (DNR) codes or treatment discontinuation, e.g., withholding or withdrawal medical care in a reliable way. This cohort represents the first months of the pandemic in the Netherlands, during which an understandable emphasis was put on patient care rather than on reporting DNR codes in the patient records. However, since mortality is strongly influenced by the decision to discontinue treatment, this may have interfered with our findings [46]. Second, there is an intercountry difference in the willingness of patients to consider ICU admission. Compared to other countries, doctors as well as patients seem to be more reluctant to proceed with ICU admission when the situation worsens [47]. This could result in a selection bias and should be considered when extrapolating these results to other countries with a more liberal ICU admission policy. In fact, we expect the association of age with mortality to be even stronger in those countries. As mentioned above, we could also not collect data on the frailty, which is another important limitation. In addition, the PRoVENT–COVID trial was conducted in the first three months of the national outbreak in the Netherlands. Due to the introduction of e.g., dexamethasone and improved prophylaxis against venous thromboembolic events, and also the vaccination program, current ICU cohorts might be different.

CONCLUSIONS

In this cohort of critically ill invasively ventilated COVID–19 patients, there were no meaningful differences in ventilator management between groups based on age quartiles. The use of adjunctive therapies for refractory hypoxemia was not affected by age, except for use of NMBAs that decreased with higher age. Older patients developed complications more often, had a longer duration of ventilation and higher mortality rates.

Abbreviations

APACHE: Acute Physiology and Chronic Health Evaluation; ARDS: Acute Respiratory Distress Syndrome; BMI: Body Mass Index; $EtCO_2$: End tidal Carbon dioxide; COPD: Chronic Obstructive Pulmonary Disease; COVID-19: coronavirus disease 2019; Crs: Respiratory system compliance; ECMO: Extracorporeal membrane oxygenation; FiO_2: Inspired Oxygen Fraction; PaO2: Partial Pressure of Oxygen; PEEP: Positive End-Expiratory Pressure; PBW: Predicted Body Weight; SAPS: The Simplified Acute Physiology Score; SOFA: Sequential Organ Failure; V_T: Tidal Volume; ΔP: Driving Pressure.

AUTHOR CONTRIBUTIONS

LH, ASN, FP and MJS designed the study and wrote the protocol. LH and ASN analyzed the data. LH and MJS drafted the manuscript. All authors made a substantial contribution to data interpretation. All authors read and approved the manuscript.

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The PRoVENT-COVID investigators (see supplement).

CONFLICTS OF INTEREST

Ary Serpa Neto reports personal fees from Dräger, outside of the submitted work. Marcus Schultz reports personal fees from Hamilton and Xenios/Novalung, outside of the submitted work. The other authors declare no conflicts of interest.

FUNDING

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REFERENCES

1. Medicine JHUa. COVID-19 Dashboard by the Center for Systems Science and Engineering. 2021. https://coronavirus.jhu.edu/map.html

2. WHO. World Health Organization Coronavirus (COVID-19) Dashboard. 2021. https://covid19.who.int/

3. Wiersinga WJ, Rhodes A, Cheng AC, Peacock SJ, Prescott HC. Pathophysiology, Transmission, Diagnosis, and Treatment of Coronavirus Disease 2019 (COVID-19): A Review. JAMA. 2020; 324:782–93. https://doi.org/10.1001/jama.2020.12839 PMID:32648899

4. Sharma G, Goodwin J. Effect of aging on respiratory system physiology and immunology. Clin Interv Aging. 2006; 1:253–60. https://doi.org/10.2147/ciia.2006.1.3.253 PMID:18046878

5. Verbeken EK, Cauberghs M, Mertens I, Clement J, Lauweryns JM, Van de Woestijne KP. The senile lung. Comparison with normal and emphysematous lungs. 1. Structural aspects. Chest. 1992; 101:793–9. https://doi.org/10.1378/chest.101.3.793 PMID:1541148

6. Gee MH, Gottlieb JE, Albertine KH, Kubis JM, Peters SP, Fish JE. Physiology of aging related to outcome in the adult respiratory distress syndrome. J Appl Physiol (1985). 1990; 69:822–9. https://doi.org/10.1152/jappl.1990.69.3.822 PMID:2246169

7. Antonia Koutsoukou MK, Orfanos S, Rovina N, Dimitrakopoulou C, Kotanidou A, Koutsoukou A. ARDS in Aged Patients: Respiratory System Mechanics and Outcome. Health Sci J, 2017; 11:498. https://doi.org/10.21767/1791-809X.1000498

8. Salzberger B, Buder F, Lampl B, Ehrenstein B, Hitzenbichler F, Hanses F. Epidemiologie von SARS-CoV-2-Infektion und COVID-19 [Epidemiology of SARS-CoV-2 infection and COVID-19]. Internist (Berl). 2020; 61:782–8. https://doi.org/10.1007/s00108-020-00834-9 PMID:32548652

9. Yang J, Zheng Y, Gou X, Pu K, Chen Z, Guo Q, Ji R, Wang H, Wang Y, Zhou Y. Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. Int J Infect Dis. 2020; 94:91–5. https://doi.org/10.1016/j.ijid.2020.03.017 PMID:32173574

10. Fang X, Li S, Yu H, Wang P, Zhang Y, Chen Z, Li Y, Cheng L, Li W, Jia H, Ma X. Epidemiological, comorbidity factors with severity and prognosis of COVID-19: a systematic review and meta-analysis. Aging (Albany NY). 2020; 12:12493–503. https://doi.org/10.18632/aging.103579 PMID:32658868

11. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H, Wang T, Guo W, Chen J, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091. https://doi.org/10.1136/bmj.m1091 PMID:32217556

12. Wu Z, McGoogan JM. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. JAMA. 2020; 323:1239–42. https://doi.org/10.1001/jama.2020.2648 PMID:32091533

13. Grasselli G, Greco M, Zanella A, Albano G, Antonelli M, Bellani G, Bonanomi E, Cabrini L, Carlesso E, Castelli G, Cattaneo S, Cereda D, Colombo S, et al, and COVID-19 Lombardy ICU Network. Risk Factors Associated With Mortality Among Patients With COVID-19 in Intensive Care Units in Lombardy, Italy. JAMA Intern Med. 2020; 180:1345–55. https://doi.org/10.1001/jamainternmed.2020.3539 PMID:32667669

14. Tinetti ME, Fried TR, Boyd CM. Designing health care for the most common chronic condition--multimorbidity. JAMA. 2012; 307:2493–4. https://doi.org/10.1001/jama.2012.5265 PMID:22797447

15. Hirani V, Naganathan V, Blyth F, Le Couteur DG, Gnjidic D, Stanaway FF, Seibel MJ, Waite LM, Handelsman DJ, Cumming RG. Multiple, but not traditional risk factors predict mortality in older people: the Concord Health and Ageing in Men Project. Age (Dordr). 2014; 36:9732. https://doi.org/10.1007/s11357-014-9732-2 PMID:25403157

16. Macaulay R, Akbar AN, Henson SM. The role of the T cell in age-related inflammation. Age (Dordr). 2013; 35:563–72. https://doi.org/10.1007/s11357-012-9381-2 PMID:22252437

17. Hoogendijk EO, Afilalo J, Ensrud KE, Kowal P, Onder G, Fried LP. Frailty: implications for clinical practice and public health. Lancet. 2019; 394:1365–75. https://doi.org/10.1016/S0140-6736(19)31786-6 PMID:31609228

18. Botta M, Tsonas AM, Pillay J, Boers LS, Algera AG, Bos LD, Dongelmans DA, Hollmann MW, Horn J, Vlaar AP,

Schultz MJ, Neto AS, Paulus F, and PRoVENT-COVID Collaborative Group. Ventilation management and clinical outcomes in invasively ventilated patients with COVID-19 (PRoVENT-COVID): a national, multicentre, observational cohort study. Lancet Respir Med. 2021; 9:139–48.
https://doi.org/10.1016/S2213-2600(20)30459-8
PMID:33169671

19. Boers NS, Botta M, Tsonas AM, Algera AG, Pillay J, Dongelmans DA, Horn J, Vlaar AP, Hollmann MW, Bos LD, Paulus F, Neto AS, Schultz MJ, and PRoVENT-COVID investigators†. PRactice of VENTilation in Patients with Novel Coronavirus Disease (PRoVENT-COVID): rationale and protocol for a national multicenter observational study in The Netherlands. Ann Transl Med. 2020; 8:1251.
https://doi.org/10.21037/atm-20-5107
PMID:33178783

20. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, Camporota L, Slutsky AS, and ARDS Definition Task Force. Acute respiratory distress syndrome: the Berlin Definition. JAMA. 2012; 307:2526–33.
https://doi.org/10.1001/jama.2012.5669
PMID:22797452

21. Schavemaker R, Schultz MJ, Lagrand WK, van Slobbe-Bijlsma ER, Serpa Neto A, Paulus F, and The PRoVENT-Covid Collaborative Group. Associations of Body Mass Index with Ventilation Management and Clinical Outcomes in Invasively Ventilated Patients with ARDS Related to COVID-19-Insights from the PRoVENT-COVID Study. J Clin Med. 2021; 10:1176.
https://doi.org/10.3390/jcm10061176
PMID:33799735

22. COVID-ICU Group on behalf of the REVA Network and the COVID-ICU Investigators. Clinical characteristics and day-90 outcomes of 4244 critically ill adults with COVID-19: a prospective cohort study. Intensive Care Med. 2021; 47:60–73.
https://doi.org/10.1007/s00134-020-06294-x
PMID:33211135

23. Richards-Belle A, Orzechowska I, Gould DW, Thomas K, Doidge JC, Mouncey PR, Christian MD, Shankar-Hari M, Harrison DA, Rowan KM, and ICNARC COVID-19 Team. COVID-19 in critical care: epidemiology of the first epidemic wave across England, Wales and Northern Ireland. Intensive Care Med. 2020; 46:2035–47.
https://doi.org/10.1007/s00134-020-06267-0
PMID:33034689

24. Slutsky AS, Ranieri VM. Ventilator-induced lung injury. N Engl J Med. 2013; 369:2126–36.
https://doi.org/10.1056/NEJMra1208707
PMID:24283226

25. Nunn JF, Hill DW. Respiratory dead space and arterial to end-tidal carbon dioxide tension difference in anesthetized man. J Appl Physiol. 1960; 15:383–9.
https://doi.org/10.1152/jappl.1960.15.3.383
PMID:14427915

26. St Croix CM, Cunningham DA, Kowalchuk JM, McConnell AK, Kirby AS, Scheuermann BW, Petrella RJ, Paterson DH. Estimation of arterial PCO2 in the elderly. J Appl Physiol (1985). 1995; 79:2086–93.
https://doi.org/10.1152/jappl.1995.79.6.2086
PMID:8847277

27. Satoh K, Ohashi A, Kumagai M, Sato M, Kuji A, Joh S. Evaluation of Differences between $PaCO_2$ and $ETCO_2$ by Age as Measured during General Anesthesia with Patients in a Supine Position. Journal of Anesthesiology. 2015; 2015:710537.
https://doi.org/10.1155/2015/710537

28. De Jong A, Chanques G, Jaber S. Mechanical ventilation in obese ICU patients: from intubation to extubation. Crit Care. 2017; 21:63.
https://doi.org/10.1186/s13054-017-1641-1
PMID:28320439

29. Lee LA, Athanassoglou V, Pandit JJ. Neuromuscular blockade in the elderly patient. J Pain Res. 2016; 9:437–44.
https://doi.org/10.2147/JPR.S85183 PMID:27382330

30. Arroliga AC, Thompson BT, Ancukiewicz M, Gonzales JP, Guntupalli KK, Park PK, Wiedemann HP, Anzueto A, and Acute Respiratory Distress Syndrome Network. Use of sedatives, opioids, and neuromuscular blocking agents in patients with acute lung injury and acute respiratory distress syndrome. Crit Care Med. 2008; 36:1083–8.
https://doi.org/10.1097/CCM.0B013E3181653895
PMID:18401254

31. Ball M, Moore RA, Fisher A, McQuay HJ, Allen MC, Sear J. Renal failure and the use of morphine in intensive care. The Lancet. 1985; 325:784–6.
https://doi.org/10.1016/S0140-6736(85)91448-5

32. deBacker J, Hart N, Fan E. Neuromuscular Blockade in the 21st Century Management of the Critically Ill Patient. Chest. 2017; 151:697–706.
https://doi.org/10.1016/j.chest.2016.10.040
PMID:27818334

33. Dhingra R, Vasan RS. Age as a risk factor. Med Clin North Am. 2012; 96:87–91.
https://doi.org/10.1016/j.mcna.2011.11.003
PMID:22391253

34. Rodgers JL, Jones J, Bolleddu SI, Vanthenapalli S, Rodgers LE, Shah K, Karia K, Panguluri SK. Cardiovascular Risks Associated with Gender and Aging. J Cardiovasc Dev Dis. 2019; 6:19.

https://doi.org/10.3390/jcdd6020019
PMID:31035613

35. Chao CT, Wang J, Wu HY, Huang JW, Chien KL. Age modifies the risk factor profiles for acute kidney injury among recently diagnosed type 2 diabetic patients: a population-based study. Geroscience. 2018; 40:201–17.
https://doi.org/10.1007/s11357-018-0013-3
PMID:29488059

36. Schmitt R, Coca S, Kanbay M, Tinetti ME, Cantley LG, Parikh CR. Recovery of kidney function after acute kidney injury in the elderly: a systematic review and meta-analysis. Am J Kidney Dis. 2008; 52:262–71.
https://doi.org/10.1053/j.ajkd.2008.03.005
PMID:18511164

37. Ely EW, Wheeler AP, Thompson BT, Ancukiewicz M, Steinberg KP, Bernard GR. Recovery rate and prognosis in older persons who develop acute lung injury and the acute respiratory distress syndrome. Ann Intern Med. 2002; 136:25–36.
https://doi.org/10.7326/0003-4819-136-1-200201010-00007 PMID:11777361

38. Brun-Buisson C, Minelli C, Bertolini G, Brazzi L, Pimentel J, Lewandowski K, Bion J, Romand JA, Villar J, Thorsteinsson A, Damas P, Armaganidis A, Lemaire F, and ALIVE Study Group. Epidemiology and outcome of acute lung injury in European intensive care units. Results from the ALIVE study. Intensive Care Med. 2004; 30:51–61.
https://doi.org/10.1007/s00134-003-2022-6
PMID:14569423

39. Quality of Life After Mechanized Ventilation in the Elderly Study Investigators. 2-month mortality and functional status of critically ill adult patients receiving prolonged mechanical ventilation. Chest. 2002; 121:549–58.
https://doi.org/10.1378/chest.121.2.549
PMID:11834671

40. Santa Cruz R, Villarejo F, Figueroa A, Cortés-Jofré M, Gagliardi J, Navarrete M. Mortality in Critically Ill Elderly Individuals Receiving Mechanical Ventilation. Respir Care. 2019; 64:473–83.
https://doi.org/10.4187/respcare.06586
PMID:30944228

41. Jung C, Fjølner J, Bruno RR, Wernly B, Artigas A, Bollen Pinto B, Schefold JC, Wolff G, Kelm M, Beil M, Sviri S, van Heerden PV, Szczeklik W, et al, and COVIP Study Group. Differences in mortality in critically ill elderly patients during the second COVID-19 surge in Europe. Crit Care. 2021; 25:344.
https://doi.org/10.1186/s13054-021-03739-7
PMID:34556171

42. Leoni ML, Lombardelli L, Colombi D, Bignami EG, Pergolotti B, Repetti F, Villani M, Bellini V, Rossi T, Halasz G, Caprioli S, Micheli F, Nolli M. Prediction of 28-day mortality in critically ill patients with COVID-19: Development and internal validation of a clinical prediction model. PLoS One. 2021; 16:e0254550.
https://doi.org/10.1371/journal.pone.0254550
PMID:34255793

43. Guillon A, Laurent E, Godillon L, Kimmoun A, Grammatico-Guillon L. Long-term mortality of elderly patients after intensive care unit admission for COVID-19. Intensive Care Med. 2021; 47:710–2.
https://doi.org/10.1007/s00134-021-06399-x
PMID:33844045

44. Jung C, Flaatten H, Fjølner J, Bruno RR, Wernly B, Artigas A, Bollen Pinto B, Schefold JC, Wolff G, Kelm M, Beil M, Sviri S, van Heerden PV, et al, and COVIP study group. The impact of frailty on survival in elderly intensive care patients with COVID-19: the COVIP study. Crit Care. 2021; 25:149.
https://doi.org/10.1186/s13054-021-03551-3
PMID:33874987

45. Flaatten H, de Lange DW, Artigas A, Bin D, Moreno R, Christensen S, Joynt GM, Bagshaw SM, Sprung CL, Benoit D, Soares M, Guidet B. The status of intensive care medicine research and a future agenda for very old patients in the ICU. Intensive Care Med. 2017; 43:1319–28.
https://doi.org/10.1007/s00134-017-4718-z
PMID:28238055

46. Flaatten H, Guidet B, de Lange DW, Beil M, Leaver SK, Fjølner J, van Heerden PV, Sigal S, Szczeklik W, Jung C. The importance of revealing data on limitation of life sustaining therapy in critical ill elderly Covid-19 patients. J Crit Care. 2022; 67:147–8.
https://doi.org/10.1016/j.jcrc.2021.10.024
PMID:34781100

47. Haas LE, Karakus A, Holman R, Cihangir S, Reidinga AC, de Keizer NF. Trends in hospital and intensive care admissions in the Netherlands attributable to the very elderly in an ageing population. Crit Care. 2015; 19:353.
https://doi.org/10.1186/s13054-015-1061-z
PMID:26423744

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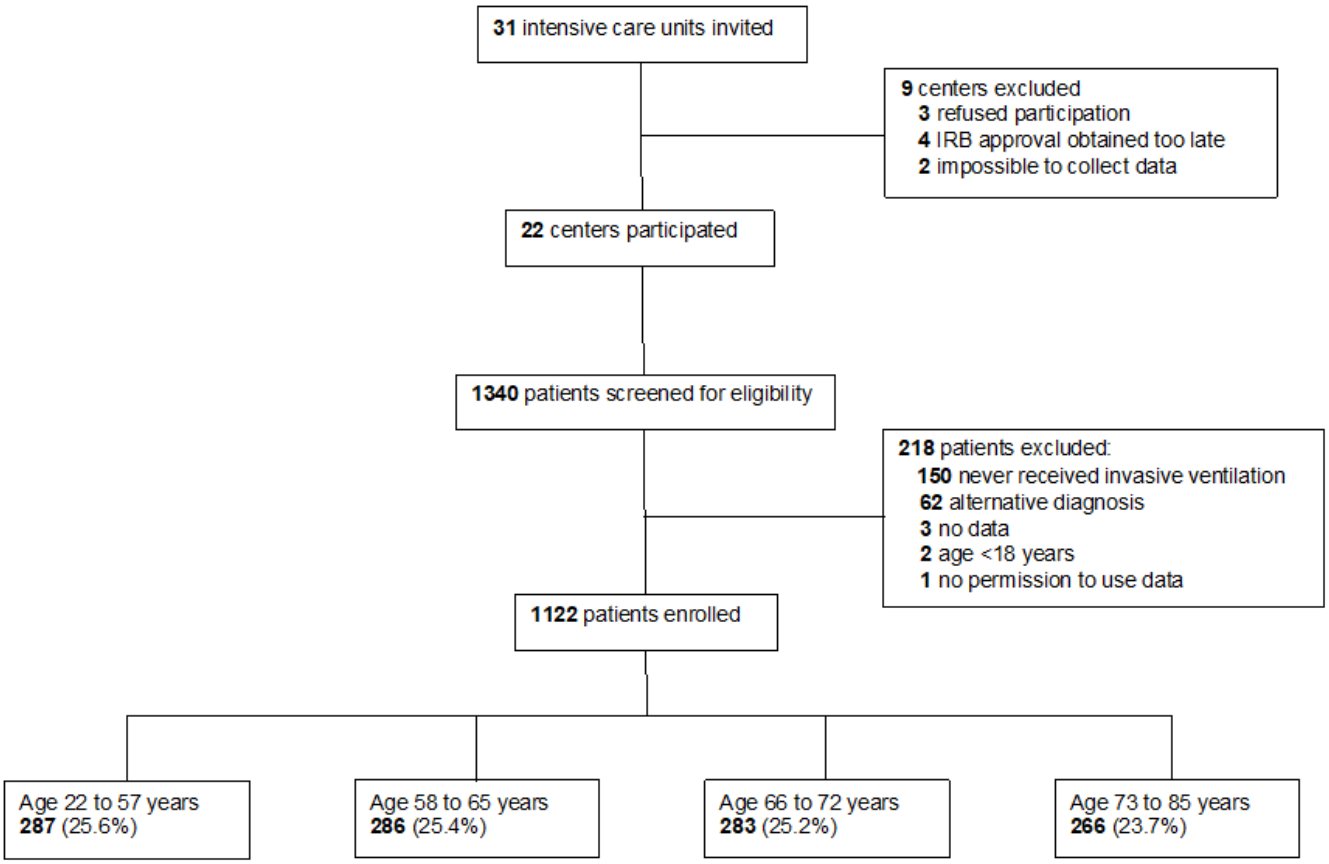
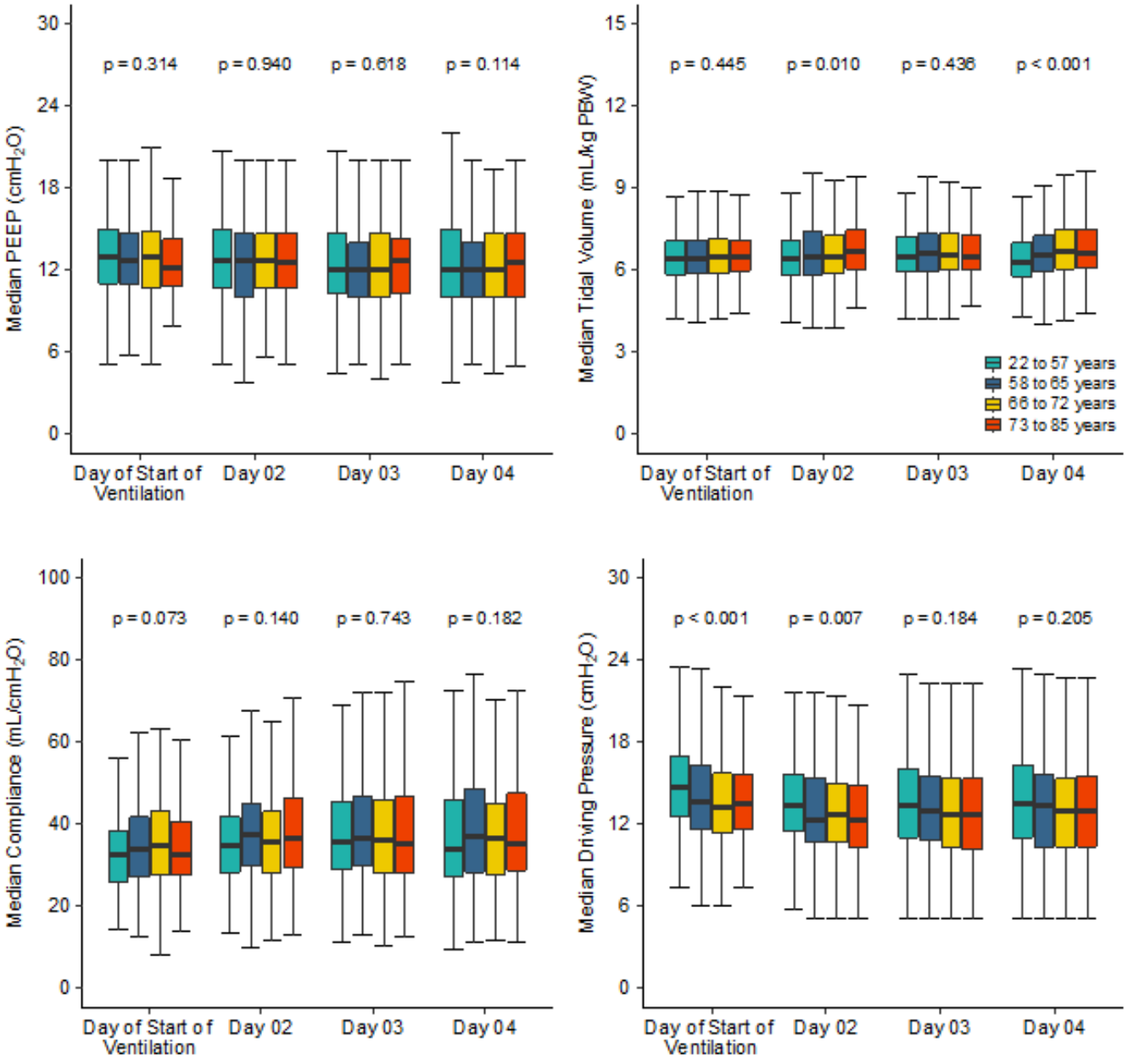
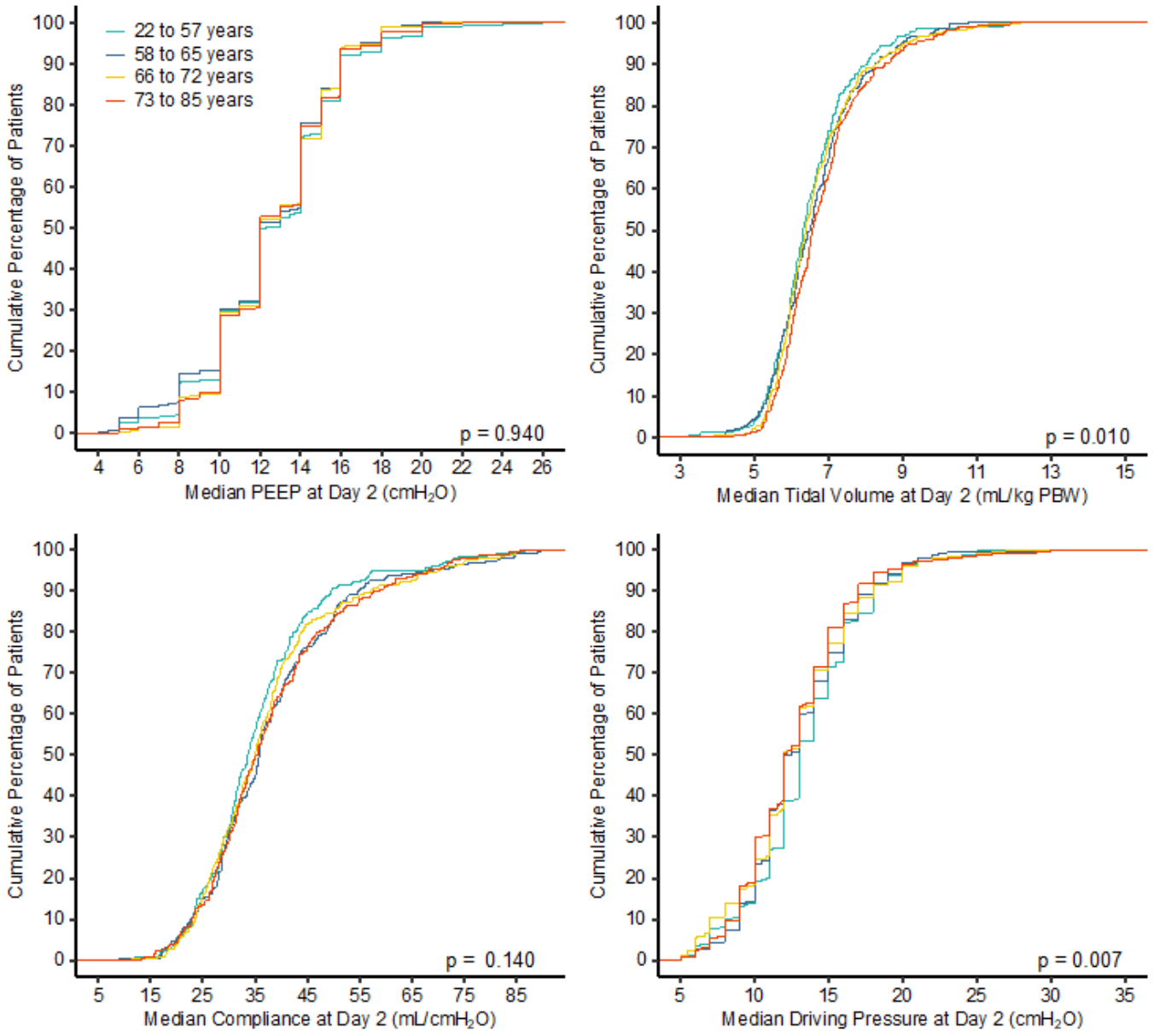
Supplementary Figures



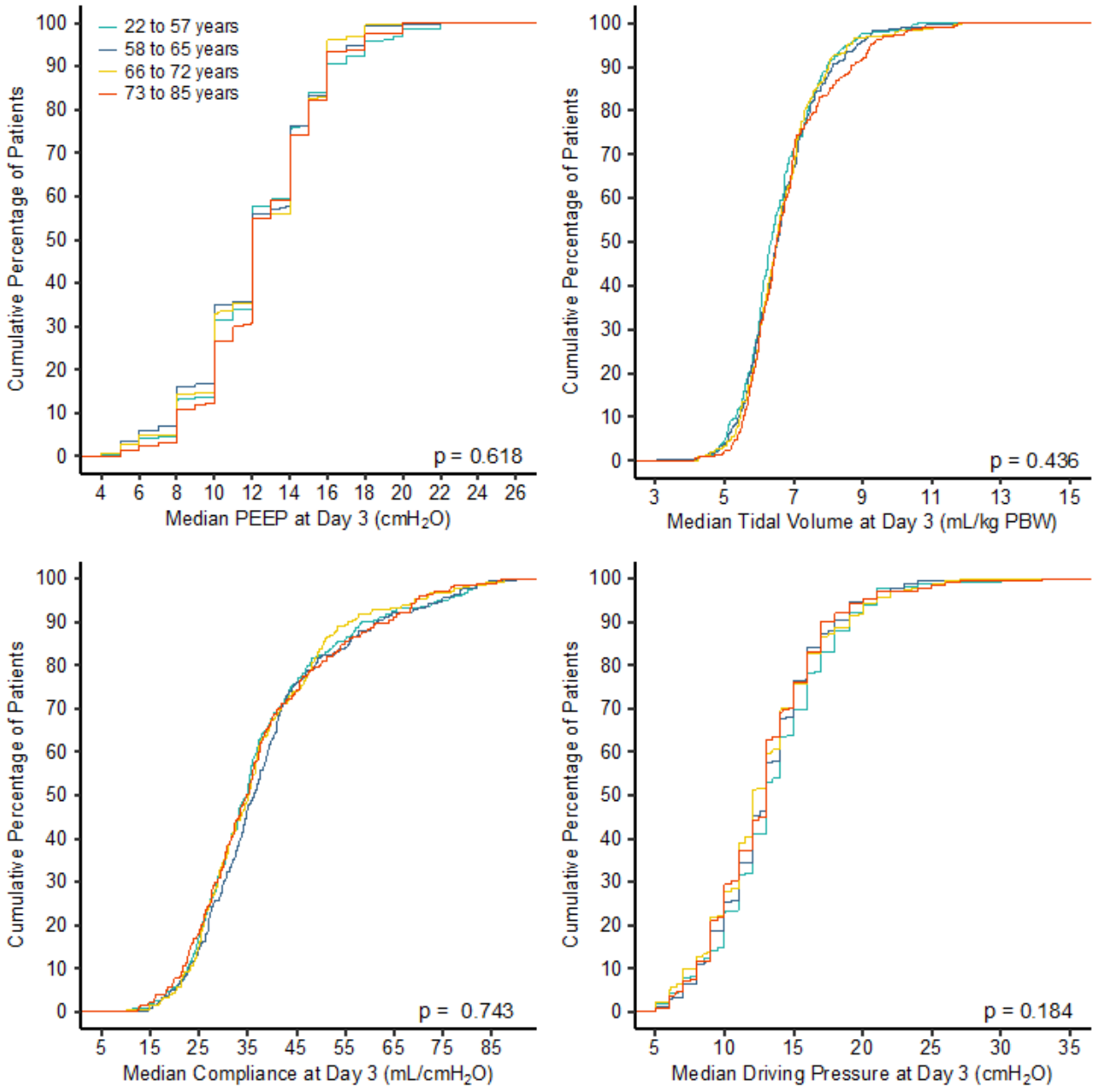
Supplementary Figure 1. CONSORT flow chart of study population.



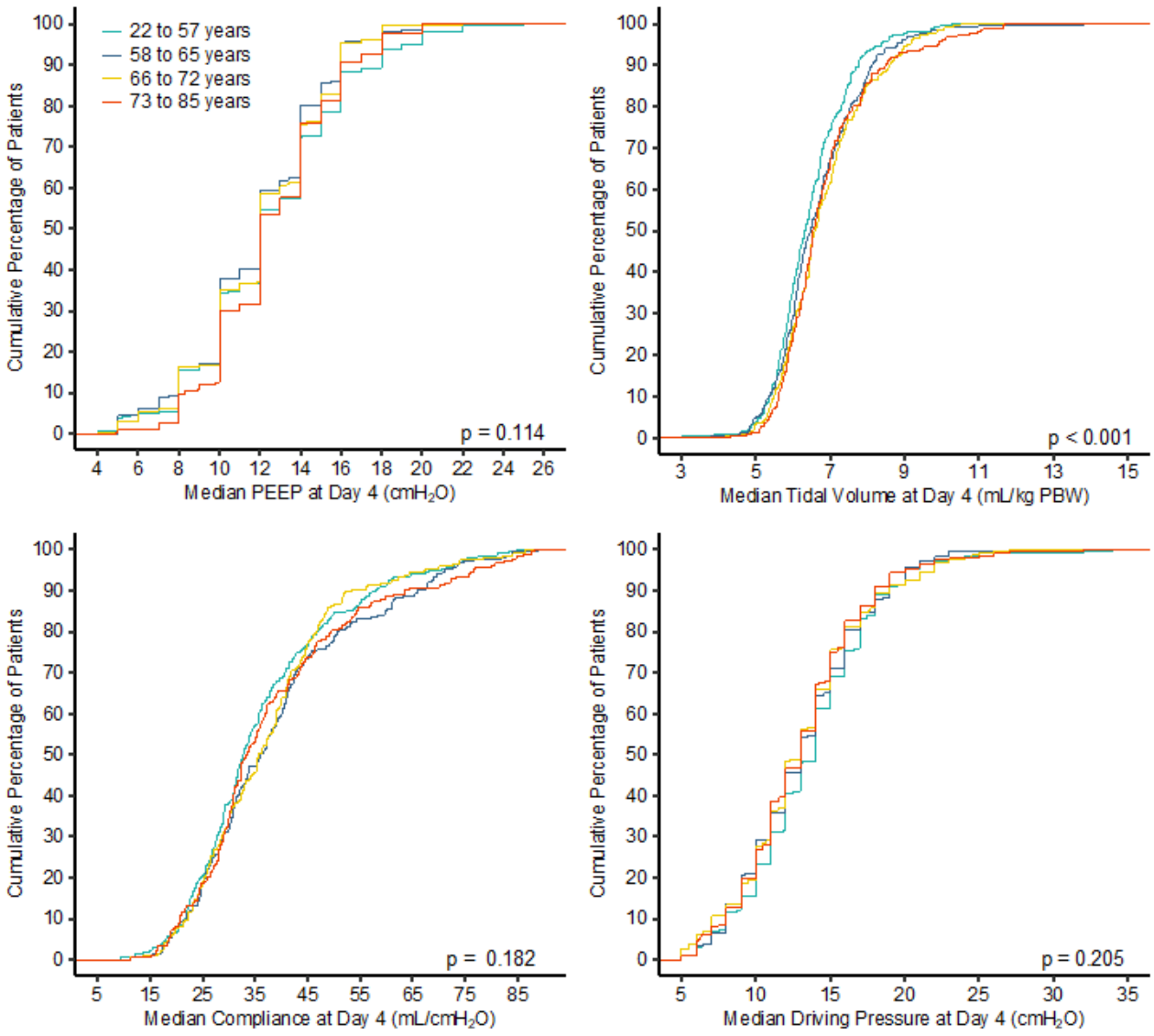
Supplementary Figure 2. Ventilatory variables in the first four days of ventilation. Boxes represent median and interquartile range. Median was calculated from the mean value of three or four measurements available on each day of ventilation. The Kruskal-Wallis test is used to calculate p-values.



Supplementary Figure 3. Cumulative frequency distribution of median PEEP to tidal volume to compliance and driving pressure on the second day of ventilation. Mean values were calculated from three measurements available on the second day of ventilation. The Kruskal-Wallis test is used to calculate p-values.



Supplementary Figure 4. Cumulative frequency distribution of median PEEP to tidal volume to compliance and driving pressure on the third day of ventilation. Mean values were calculated from three measurements available on the third day of ventilation. The Kruskal-Wallis test is used to calculate p-values.



Supplementary Figure 5. Cumulative frequency distribution of median PEEP to tidal volume to compliance and driving pressure on the fourth day of ventilation. Mean values were calculated from three measurements available on the fourth day of ventilation. The Kruskal-Wallis test was is to calculate p-values.

Supplementary Tables

Supplementary Table 1. Characteristics of mechanical ventilation in the first four days of ventilation.

	Age 22 to 57 years (*n* = 287)	Age 58 to 65 years (*n* = 286)	Age 66 to 72 years (*n* = 283)	Age 73 to 85 years (*n* = 266)	*P* value
Tidal volume, mL/kg PBW					
Day 1	6.4 [5.8 to 7.0]	6.4 [5.9 to 7.1]	6.5 [5.9 to 7.1]	6.5 [6.0 to 7.1]	0.445
Day 2	6.4 [5.8 to 7.1]	6.5 [5.8 to 7.4]	6.5 [5.9 to 7.3]	6.7 [6.0 to 7.4]	0.010
Day 3	6.5 [5.9 to 7.2]	6.6 [5.9 to 7.3]	6.5 [6.0 to 7.4]	6.5 [6.0 to 7.2]	0.437
Day 4	6.3 [5.8 to 7.0]	6.6 [5.9 to 7.2]	6.7 [6.0 to 7.5]	6.6 [6.1 to 7.5]	<0.001
PEEP, cmH_2O					
Day 1	13.0 [11.0 to 15.0]	12.7 [11.0 to 14.6]	13.0 [10.7 to 14.8]	12.2 [10.8 to 14.2]	0.314
Day 2	12.7 [10.7 to 15.0]	12.7 [10.0 to 14.7]	12.7 [10.7 to 14.7]	12.6 [10.7 to 14.7]	0.940
Day 3	12.0 [10.3 to 14.7]	12.0 [10.0 to 14.0]	12.0 [10.0 to 14.7]	12.7 [10.3 to 14.3]	0.618
Day 4	12.0 [10.0 to 15.0]	12.0 [10.0 to 14.0]	12.0 [10.0 to 14.7]	12.5 [10.0 to 14.7]	0.114
Driving pressure, cmH_2O					
Day 1	14.7 [12.5 to 17.0]	13.8 [11.7 to 16.3]	13.2 [11.3 to 15.7]	13.5 [11.6 to 15.7]	<0.001
Day 2	13.3 [11.4 to 15.7]	12.3 [10.7 to 15.3]	12.7 [10.7 to 15.0]	12.4 [10.3 to 15.0]	0.007
Day 3	13.3 [11.0 to 16.0]	13.0 [10.8 to 15.5]	12.7 [10.3 to 15.3]	12.7 [10.1 to 15.3]	0.184
Day 4	13.7 [11.0 to 16.3]	13.3 [10.3 to 15.7]	13.0 [10.3 to 15.3]	13.0 [10.3 to 15.5]	0.205
Compliance, mL/cmH_2O					
Day 1	32.4 [25.9 to 38.3]	33.8 [27.1 to 41.7]	34.7 [27.7 to 43.3]	32.6 [27.3 to 40.7]	0.073
Day 2	34.9 [28.4 to 42.2]	37.5 [29.8 to 45.4]	35.7 [28.3 to 43.5]	36.6 [29.6 to 46.7]	0.140
Day 3	35.5 [28.9 to 45.4]	36.5 [29.6 to 47.0]	36.0 [28.2 to 47.1]	35.4 [27.9 to 47.5]	0.743
Day 4	33.9 [26.9 to 45.6]	36.8 [28.6 to 49.1]	37.0 [27.9 to 47.0]	35.3 [28.7 to 49.4]	0.182
Peak pressure, cmH_2O					
Day 1	27.7 [25.0 to 30.8]	26.7 [23.3 to 30.0]	26.0 [23.3 to 29.2]	26.2 [23.6 to 29.0]	<0.001
Day 2	26.3 [23.0 to 29.7]	25.3 [22.3 to 29.0]	25.7 [22.0 to 28.3]	25.3 [22.0 to 28.3]	0.102
Day 3	26.0 [22.0 to 29.7]	25.7 [21.3 to 28.5]	25.3 [20.7 to 28.8]	25.3 [21.3 to 29.0]	0.362
Day 4	26.3 [22.0 to 29.7]	25.3 [20.4 to 28.9]	25.3 [20.7 to 28.7]	25.3 [22.0 to 29.3]	0.145
Mechanical power, J/min					
Day 1	19.2 [16.0 to 23.7]	19.3 [15.9 to 23.1]	17.9 [14.7 to 22.3]	17.2 [14.6 to 20.9]	<0.001
Day 2	18.8 [15.7 to 23.5]	19.1 [15.8 to 23.2]	18.6 [14.6 to 22.9]	18.1 [14.4 to 22.3]	0.237
Day 3	19.2 [15.1 to 24.1]	19.7 [15.4 to 23.8]	18.8 [14.9 to 22.6]	18.7 [15.2 to 23.1]	0.619
Day 4	19.2 [15.9 to 24.0]	19.5 [15.2 to 23.9]	19.3 [15.1 to 23.3]	19.3 [16.3 to 23.5]	0.882
$PaCO_2$, mmHg					
Day 1	42.9 [38.3 to 48.4]	44.6 [39.8 to 49.5]	46.1 [39.9 to 52.0]	45.0 [39.1 to 50.9]	0.002
Day 2	44.5 [40.0 to 49.5]	46.6 [41.8 to 52.5]	45.4 [42.0 to 53.3]	45.5 [40.6 to 51.8]	0.060
Day 3	46.8 [42.5 to 54.8]	48.3 [43.4 to 53.8]	47.3 [42.8 to 55.3]	47.3 [41.8 to 54.0]	0.483
Day 4	48.5 [43.3 to 55.3]	49.3 [44.5 to 54.3]	48.8 [43.8 to 56.0]	48.6 [42.5 to 54.3]	0.724
$EtCO_2$, mmHg					
Day 1	38.0 [33.8 to 43.8]	37.7 [33.3 to 42.8]	36.3 [31.9 to 42.0]	35.3 [31.6 to 39.9]	<0.001
Day 2	39.8 [35.5 to 44.3]	38.6 [34.8 to 44.3]	36.8 [32.2 to 41.3]	36.8 [31.8 to 41.4]	<0.001
Day 3	41.0 [36.3 to 46.5]	38.8 [34.5 to 43.0]	37.5 [33.3 to 42.5]	36.5 [32.8 to 42.7]	<0.001
Day 4	42.3 [37.0 to 49.0]	38.5 [35.0 to 44.3]	37.5 [32.2 to 42.8]	37.5 [33.0 to 42.5]	<0.001
FiO_2					
Day 1	0.6 [0.5 to 0.7]	0.6 [0.5 to 0.7]	0.6 [0.5 to 0.7]	0.6 [0.5 to 0.7]	0.286
Day 2	0.4 [0.4 to 0.5]	0.4 [0.4 to 0.5]	0.4 [0.4 to 0.5]	0.5 [0.4 to 0.5]	0.269
Day 3	0.4 [0.4 to 0.5]	0.4 [0.4 to 0.5]	0.4 [0.4 to 0.5]	0.4 [0.4 to 0.5]	0.750
Day 4	0.4 [0.4 to 0.5]	0.4 [0.4 to 0.5]	0.5 [0.4 to 0.6]	0.5 [0.4 to 0.6]	0.294
PaO_2, mmHg					
Day 1	81.0 [71.5 to 99.3]	78.7 [71.3 to 93.4]	82.4 [72.7 to 95.4]	83.3 [75.0 to 96.0]	0.018
Day 2	75.0 [69.3 to 86.3]	75.3 [69.1 to 84.7]	75.5 [69.8 to 84.5]	76.5 [69.7 to 84.5]	0.782
Day 3	72.5 [67.1 to 82.1]	72.3 [66.0 to 80.8]	74.5 [67.4 to 81.3]	73.8 [67.5 to 81.0]	0.443
Day 4	72.0 [66.0 to 80.3]	70.8 [64.9 to 78.3]	72.3 [66.1 to 79.3]	73.1 [68.0 to 80.3]	0.120

Supplementary Table 2. Posthoc dunn test for paired comparison for patient outcomes.

Tracheostomy		**Age 22 to 57 years**	**Age 58 to 65 years**	**Age 66 to 72 years**
Age 58 to 65 years	Z test statistic	-2.992		
	P value	0.008		
Age 66 to 72 years	Z test statistic	-1.504	1.478	
	P value	0.397	0.148	
Age 73 to 85 years	Z test statistic	-1.433	1.508	0.050
	P value	0.455	0.395	1.000
Myocardial infarction		**Age 22 to 57 years**	**Age 58 to 65 years**	**Age 66 to 72 years**
Age 58 to 65 years	Z test statistic	0.703		
	P value	1.000		
Age 66 to 72 years	Z test statistic	-1.788	-2.487	
	P value	0.221	0.039	
Age 73 to 85 years	Z test statistic	-1.916	-2.605	-0.156
	P value	0.166	0.028	1.000
Acute Kidney injury		**Age 22 to 57 years**	**Age 58 to 65 years**	**Age 66 to 72 years**
Age 58 to 65 years	Z test statistic	-4.358		
	P value	<0.001		
Age 66 to 72 years	Z test statistic	-3.315	1.025	
	P value	0.003	0.916	
Age 73 to 85 years	Z test statistic	-5.242	-0.963	-1.966
	P value	<0.001	1.000	0.148
Need for renal replacement therapy		**Age 22 to 57 years**	**Age 58 to 65 years**	**Age 66 to 72 years**
Age 58 to 65 years	Z test statistic	-2.936		
	P value	0.010		
Age 66 to 72 years	Z test statistic	-2.454	0.474	
	P value	0.042	1.000	
Age 73 to 85 years	Z test statistic	-2.121	0.761	0.293
	P value	0.102	1.000	1.000
Use of neuromuscular blocking agents		**Age 22 to 57 years**	**Age 58 to 65 years**	**Age 66 to 72 years**
Age 58 to 65 years	Z test statistic	1.881		
	P value	0.180		
Age 66 to 72 years	Z test statistic	1.083	-0.792	
	P value	0.837	1.000	
Age 73 to 85 years	Z test statistic	3.588	1.740	2.514
	P value	0.001	0.246	0.036
Ventilator-free days at day 28		**Age 22 to 57 years**	**Age 58 to 65 years**	**Age 66 to 72 years**
Age 58 to 65 years	Z test statistic	4.488		
	P value	<0.001		
Age 66 to 72 years	Z test statistic	6.9400	2.446	
	P value	<0.001	0.043	
Age 73 to 85 years	Z test statistic	9.309	4.855	2.435
	P value	<0.001	<0.001	0.045

Supplementary Table 3. Multivariable assessment of factors associated with 28-day and 90-day mortality.

	28-day mortality		90-day mortality	
	Hazard ratio (95% CI)	**P value**	**Hazard ratio (95% CI)**	**P value**
Age category				
Age 22 to 57 years	1 (reference)		1 (reference)	
Age 58 to 65 years	1.37 (0.89 to 2.11)	0.150	1.49 (1.00 to 2.23)	0.050
Age 66 to 72 years	2.16 (1.43 to 3.25)	<0.001	2.32 (1.59 to 3.40)	<0.001
Age 73 to 85 years	3.35 (2.24 to 5.01)	<0.001	4.05 (2.77 to 5.93)	<0.001
Demographic characteristics				
Male gender	1.16 (0.88 to 1.52)	0.290	1.25 (0.96 to 1.62)	0.093
Body-mass index to kg/m^2	0.97 (0.85 to 1.10)	0.630	1.00 (0.90 to 1.11)	0.980
Hypertension	1.32 (1.02 to 1.72)	0.038	1.15 (0.89 to 1.47)	0.280
Heart failure	1.15 (0.70 to 1.88)	0.570	1.10 (0.69 to 1.78)	0.680
Diabetes mellitus	1.38 (1.05 to 1.82)	0.019	1.42 (1.09 to 1.84)	0.008
Chronic kidney disease	0.98 (0.58 to 1.66)	0.940	1.17 (0.72 to 1.89)	0.520
Chronic obstructive pulmonary disease	1.53 (1.05 to 2.22)	0.028	1.51 (1.06 to 2.16)	0.023
Active hematological neoplasia	1.85 (0.80 to 4.27)	0.150	1.65 (0.76 to 3.59)	0.210
Active solid tumor	1.59 (0.84 to 2.99)	0.150	1.20 (0.64 to 2.24)	0.570
Use of angiotensin-converting enzyme inhibitor	1.00 (0.73 to 1.36)	1.000	0.83 (0.61 to 1.12)	0.220
Use of angiotensin II receptor blocker	0.91 (0.64 to 1.31)	0.620	0.89 (0.63 to 1.25)	0.490
Organ support on day 0*				
Use of vasopressor or inotropes	1.11 (0.81 to 1.51)	0.510	1.09 (0.81 to 1.46)	0.570
Fluid balance to mL	1.07 (0.96 to 1.21)	0.230	1.04 (0.93 to 1.16)	0.460
Oxygenation variables on day 0*				
PaO_2/FiO_2	0.88 (0.76 to 1.01)	0.065	0.88 (0.77 to 1.00)	0.044
Laboratory tests on day 0*				
Creatinine to µmol/L	1.00 (0.91 to 1.09)	0.980	1.02 (0.94 to 1.10)	0.620
pH	0.71 (0.62 to 0.82)	<0.001	0.73 (0.64 to 0.83)	<0.001
Vital signs on day 0*				
Mean arterial pressure to mm Hg	0.89 (0.79 to 1.01)	0.066	0.89 (0.79 to 1.00)	0.051
Heart rate to beats per minute	1.07 (0.94 to 1.22)	0.300	1.08 (0.96 to 1.22)	0.210

The models are mixed-effects models with centers as a random effect. *Median value on the first day of invasive ventilation.

Supplementary Table 4. Multivariable assessment of factors associated with hospital and ICU mortality.

	Hospital mortality		ICU mortality	
	Odds ratio (95% CI)	**P value**	**Odds ratio (95% CI)**	**P value**
Age category				
Age 22 to 57 years	1 (reference)		1 (reference)	
Age 58 to 65 years	1.67 (1.05 to 2.65)	0.030	1.63 (1.03 to 2.58)	0.037
Age 66 to 72 years	3.30 (2.08 to 5.24)	<0.001	3.04 (1.92 to 4.79)	<0.001
Age 73 to 85 years	5.35 (3.33 to 8.61)	<0.001	4.64 (2.90 to 7.42)	<0.001
Demographic characteristics				
Male gender	1.48 (1.04 to 2.09)	0.028	1.40 (1.00 to 1.96)	0.051
Body-mass index to kg/m^2	0.99 (0.85 to 1.14)	0.872	0.99 (0.85 to 1.14)	0.845
Hypertension	1.08 (0.75 to 1.54)	0.688	1.00 (0.70 to 1.41)	0.992
Heart failure	0.97 (0.48 to 1.94)	0.923	0.99 (0.50 to 1.95)	0.971
Diabetes mellitus	1.43 (1.00 to 2.05)	0.053	1.44 (1.01 to 2.06)	0.043
Chronic kidney disease	1.42 (0.67 to 3.00)	0.357	1.45 (0.70 to 2.99)	0.321
Chronic obstructive pulmonary disease	1.56 (0.91 to 2.67)	0.108	1.50 (0.89 to 2.51)	0.218
Active hematological neoplasia	2.29 (0.74 to 7.14)	0.152	2.55 (0.85 to 7.66)	0.095
Active solid tumor	1.05 (0.44 to 2.52)	0.916	1.18 (0.50 to 2.81)	0.701
Use of angiotensin-converting enzyme inhibitor	0.78 (0.51 to 1.19)	0.253	0.88 (0.58 to 1.34)	0.556
Use of angiotensin II receptor blocker	0.88 (0.53 to 1.45)	0.600	0.95 (0.58 to 1.55)	0.823
Organ support on day 0*				
Use of vasopressor or inotropes	1.15 (0.79 to 1.69)	0.465	1.16 (0.80 to 1.69)	0.435
Fluid balance to mL	1.00 (0.86 to 1.17)	0.954	1.05 (0.90 to 1.22)	0.525
Oxygenation variables on day 0*				
PaO_2/FiO_2	0.86 (0.73 to 1.03)	0.098	0.83 (0.70 to 0.98)	0.031
Laboratory tests on day 0*				
Creatinine to µmol/L	1.09 (0.92 to 1.29)	0.321	1.07 (0.92 to 1.24)	0.405
pH	0.68 (0.57 to 0.81)	<0.001	0.69 (0.59 to 0.82)	<0.001
Vital signs on day 0*				
Mean arterial pressure to mm Hg	0.86 (0.73 to 1.01)	0.062	0.87 (0.74 to 1.01)	0.069
Heart rate to beats per minute	1.10 (0.94 to 1.30)	0.245	1.07 (0.91 to 1.26)	0.392

The models are mixed-effects models with centers as a random effect. *Median value on the first day of invasive ventilation.

Research Paper

Physiological health indexes predict deterioration and mortality in patients with COVID-19: a comparative study

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ABSTRACT

Old age is a crucial risk factor for severe coronavirus disease 2019 (COVID-19), with serious or fatal outcomes disproportionately affecting older adults compared with the rest of the population. We proposed that the physiological health status and biological age, beyond the chronological age itself, could be the driving trends affecting COVID-19 severity and mortality. A total of 155 participants hospitalized with confirmed COVID-19 aged 26–94 years were recruited for the study. Four different physiological summary indices were calculated: Klemera and Doubal's biological age, PhenoAge, physiological dysregulation (PD; globally and in specific systems), and integrated albunemia. All of these indices significantly predicted the risk of death ($p < 0.01$) after adjusting for chronological age and sex. In all models, men were 2.4–4.4-times more likely to die than women. The global PD was shown to be a good predictor of deterioration, with the odds of deterioration increasing by 41.7% per 0.5-unit increase in the global PD. As for death, the odds also increased by 68.3% per 0.5-unit increase in the global PD. Our results are partly attributed to common chronic diseases that aggravate COVID-19, but they also suggest that the underlying physiological state could capture vulnerability to severe COVID-19 and serve as a tool for prognosis that would, in turn, help inpatient management.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) remains one of the main threats to public health worldwide. Owing to the clinical variability of the COVID-19 disease course, it is important to search for predictors that reliably predict the severity of this disease. The pandemic experience has shown that the greatest risks of COVID-19 severe course and unfavorable outcomes of the disease are age and aging-associated diseases; compared to the 50–60-year age group, the risk of death is 23 times higher for individuals aged > 65 years and 100 times higher for those aged > 85 years. The possible causes of aging-related disparities among severe cases of COVID-19 infection have been widely discussed in the scientific literature [1]. In addition to the most obvious explanation, which is the pronounced comorbidity among elderly patients, a hypothesis regarding the influence of immunosenescence has been proposed [2, 3]. Zhavoronkov et al. posited that aging-associated immunosenescence reduces the ability to protect humans against infection and infection causes biological damage to the body, leading to a loss of homeostasis. These factors lead to the acceleration of the aging processes and the worsening of aging-related diseases. Another significant factor in the high mortality from COVID-19 among the elderly population is the accumulation of functional deficits that occur with increasing age and frailty. It has been shown that frailty syndrome is directly related to mortality [4]. In contrast, it is well known that the rate of aging differs significantly among humans. These differences are vividly represented in both persons with early signs of aging and nonagenarians and centenarians who maintain a good physique for a long time. Thus, there is a need to develop a tool for assessing the clinical and physiological states of a person for a more accurate individual prognosis of the course of COVID-19 infection, which could become a scientific basis for making timely and effective clinical decisions. It is especially important to find and validate those predictors of a severe disease course that could predict the outcome of the disease more effectively than the chronological age.

According to existing data, various calculations for assessing physiological state and biological age can be considered promising predictors of the severity of the course of COVID-19 [5], including measures of the biological age, such as the PhenoAge (PA) and Klemera-Doubal method (KD), integrated albunemia, and physiological dysregulation. In a study by Kuo et al. based on data from the UK Biobank, accelerated aging calculated using the PA 10–14 years before the onset of the COVID-19 pandemic was associated with all-cause mortality in patients with COVID-19 [6]. Differences in the methods used to calculate the physiological states may influence their predictive power. Therefore, to determine the most informative method for assessing physiological state or biological age in relation to the prognosis of COVID-19, it is necessary to conduct comparative studies. In this study, we aimed to assess whether different multivariate metrics of physiological state could predict the outcomes of COVID-19 better than the chronological age.

MATERIALS AND METHODS

This study included men and women aged ≥ 18 years who were hospitalized in the infectious diseases department of the Hospital for War Veterans No. 3 of Moscow Health Department and diagnosed with COVID-19 by PCR testing. Diagnostics and therapy for COVID-19 were performed in accordance with the guidelines of the Ministry of Health of Russia ("Prevention, diagnosis, and treatment of a new coronavirus infection (COVID-19)), version 5 from August 4, 2020; version 6 from April 28, 2020; and version 7 from March 6, 2020. This study was approved by the Local Ethics Committee of the First Moscow State University, named after I. M. Sechenov (Sechenov University), protocol #19-20 (dated July 2, 2020), and conducted according to the guidelines of the Declaration of Helsinki.

The main purpose of this study was to measure the strength of association between the different types of physical states or biological age and the following outcomes: death, deterioration (transition to a more severe degree according to clinical guidelines), or a combination of these two. Multivariate logistic regression was applied to model the odds ratio (OR) of the outcome using sex, chronological age, and physical state or biological age (with calculators described below) as the predictors. All statistical analyses were performed using Stata version 14 software and R language. A two-sided significance level of 0.05 was used.

Different indices were used to assess the individual physiological states. The biomarkers used are listed in Table 1. First, integrated albumin (IA), a physiological emergent process notably related to inflammation [7], was calculated using the calculator provided by Cohen and the following 14 biomarkers: hemoglobin, hematocrit, MCH, mean corpuscular hemoglobin concentration (MCHC), RBC, RDW, platelets, iron, albumin to globulin ratio, calcium, CRP, alkaline phosphatase, and ALT. Second, the biological age was measured using the KD [5, 8], with eight biomarkers selected based on their availability in the dataset, their

Table 1. Biomarkers, their mean and standard deviation, measure(s) using the biomarker, and log transformation of biomarkers.

Biomarker	Mean ± SD	Measure(s)	Log-transformation for normality
Alanine transaminase (ALT, U/L)	49 ± 62	IA, PD (g)	X
Albumin (g/L)	33.8 ± 5.4	IA, KD, PA, PD (g)	
Albumin-globulin ratio	1.16 ± 0.30	IA	
Alkaline phosphatase (U/L)	223 ± 159	IA, PA	X (IA)
Aspartate transaminase (AST, U/L)	67 ± 80	PD (g)	X
Blood urea nitrogen (BUN) (mmol/L)	8.0 ± 6.0	KD	X
Calcium (mmol/L)	0.90 ± 0.40	IA	
Chronological age (years)	64 ± 15	KD, PA	
C-reactive protein (CRP)	117 ± 89	IA, KD, PA, PD (g)	X
Glucose (mmol/L)	8.0 ± 3.6	PA	
Hematocrit (%)	38.71 ± 5.89	IA	
Hemoglobin (g/L)	129 ± 18	IA, PD (g,o)	
Iron (µmol/L)	8.5 ± 5.7	IA	
Lymphocytes (%)	21 ± 15	KD, PA, PD (g,l)	
Mean corpuscular hemoglobin (MCH) (pg)	30.1 ± 2.6	IA, PD (g)	
Mean corpuscular hemoglobin concentration (MCHC) (g/dL)	33 ± 1	IA, PD (o)	
Mean corpuscular volume (MCV) (fL)	90.5 ± 6.8	PA, PD (o)	
Neutrophils (%)	72 ± 15	PD (l)	
Platelets (10^9/L)	198 ± 80	IA, KD, PD (g)	X (KD, PD)
Potassium (mmol/L)	3.9 ± 0.8	PD (g)	
Red blood cell count (RBC, 10^6/µL)	4.32 ± 0.55	IA, KD, PD (g,o)	
Red cell distribution width (RDW) (%)	14.2 ± 3.4	IA, KD, PA, PD (g,o)	X (IA, KD and PD)
Serum creatinine (mg/dL)	1.37 ± 0.96	PA	
Sodium (mmol/L)	139 ± 5	PD (g)	
Total cholesterol (mmol/L)	4.2 ± 1.2	KD	
Total protein (g/L)	64.0 ± 6.5	PD (g)	
White blood cell count (WBC) (10^9/L)	7.8 ± 4.4	PA, PD (g,l)	X (PD)

Legend:
IA, integrated albunemia; KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.
Note: units presented in this table are not necessarily the units in which biomarkers were used in the calculations; the units were adapted to measures (for example, depending on the existing formula, the reference population, etc.).

independence, and their correlation with the chronological age ($r > |0.10|$), as suggested by Levine et al. [5]: CRP, albumin, total cholesterol, blood urea nitrogen, RDW, platelets, RBC, and lymphocyte percentage. Third, PA was calculated as described by Levine et al. [9] using the albumin, creatinine, serum glucose, CRP, lymphocyte percentage, mean corpuscular volume (MCV), RDW, alkaline phosphatase, WBC, and chronological age. Finally, we calculated the physiological dysregulation (PD) using

the MD, as described elsewhere [10–13]. We selected biomarkers based on their stability in three other cohorts and calculated the PD globally and within two physiological systems:

1) The global PD included 14 biomarkers: MCH, RDW, RBC, platelets, percentage of lymphocytes, WBC, CRP, potassium, sodium, hemoglobin, albumin, ALT, AST, and total protein.
2) The PD in the oxygen transport system included the MCHC, MCV, RDW, RBC, and hemoglobin.
3) The PD in the leukopoiesis system included the percentage of neutrophils, WBC, and percentage of lymphocytes.

Due to the small sample size in our study, we used the National Health and Nutrition Examination Survey as a reference population to scale biomarkers and calculate the variance-covariance matrix [12]. The use of an external reference population was cross-validated with that of an Asian population to assess PD stability across races. As PD generally has a log-normal distribution, we used the standardized logarithm of PD (log(PD)/sd(log(PD))). Missing values for iron (67.1%), alkaline phosphatase (59.4%), calcium (2.6%), and alanine aminotransferase (0.65%) were imputed using the mouse function in R (mice package) for the IA and PA calculations. The biomarkers were log-transformed, if needed, to meet the assumptions of normality before the calculation of all measures was performed.

RESULTS

A total of 155 participants aged between 26 and 94 years from Moscow and hospitalized in the infectious disease department were recruited for this study. All patients were diagnosed with COVID-19 by polymerase chain reaction (PCR) testing and underwent treatment for confirmed COVID-19 from April 14, 2020, to June 10, 2020. Among the included participants, 47% were women (n = 73) and 53% were men (n = 82). The average age of the participants was 64 years. The average biological age calculated using the PA calculator was 75.3 years and that calculated using the KD calculator was 64 years. The other characteristics and more detailed descriptions are presented in Table 2. All other information about the cohort and measured parameters are presented in the Supplementary Data (Supplementary Tables 1–4 and Supplementary Figure 1).

First, we performed a three-factor logistic regression analysis with age and sex adjustments to evaluate the association between each cell blood count or biochemical parameters and COVID-19 outcomes (Figures 1, 2).

The most significant association was revealed for calcium level. Low calcium levels were strongly correlated with death and deterioration in patients with COVID-19 (Figure 3). In contrast, the levels of inflammatory markers, urea, liver enzymes, and glucose were increased in the patients with high deterioration and death risks.

Analyses using three-factor logistic regression models (Table 3) revealed a significant association between the risk of death and biological age/physiological state based on any of the calculators described above ($p < 0.01$) after adjusting for chronological age and sex. Thus, the odds of death increased by 68.3% per 0.5-unit increase in the global PD, by 28.5% per 0.5-unit increase in the oxygen transport-PD, by 61.9% per 0.5-unit increase in the leukopoiesis-PD, by 44.9% per 5-unit increase in the KD age, and by 62.3% per 5-unit increase in the PA. In all models, men were 2.4–4.4 times more likely to die than women. The chronological age was not a significant predictor in the KD or PA models ($p = 0.429$ and $p = 0.608$, respectively). Across all tests, the integrated albunemia was not associated with deterioration or death ($p = 0.52$ and $p = 0.43$, respectively). The dependence between the chronological age and selected metrics of the biological age or physiological state, split by death or recovery, is presented in Figure 4.

In contrast, the risk of deterioration had no significant association with PD in the oxygen transport system or PA, while the odds of deterioration increased by 41.7% per 0.5-unit increase in the global PD, by 32.9% per 0.5-unit increase in the PD in the leukopoiesis system, and by 20.4% per 5-unit increase in the KD age (Table 4). Except for the model with the PA, in which a significant association was found ($p = 0.021$), none of the other models showed any statistically significant effect of sex ($p > 0.05$). Similarly, a weakly significant association of chronological age with the odds of deterioration was revealed only for the model with KD as a predictor ($p = 0.010$), while, in all other models, chronological age was not statistically significant. The dependence between the chronological age and selected metrics of biological age or physiological state, split by deterioration, is presented in Figure 5.

As for the combined outcome (death or deterioration), the results were very similar to those for the deterioration outcome, which was expected, given that most cases of death involved deterioration (Table 5). The odds of outcome were increased by 41.7% per 0.5-unit increase in the PD global age, by 32.9% per 0.5-unit increase in the PD oxygen age, and by 20.4%

Table 2. Descriptive statistics of physiological state, chronological and biological age according to various calculators.

Parameter	Age, years	IA, u.	KD, years	PA, years	PD (g), u.	PD (o), u.	PD (l), u.
Cohort size, N	155	155	155	146	154	155	155
Mean	64.02	4.54	64.02	75.30	6.08	1.33	1.83
SD	15.24	2,47	17.31	22.75	1.00	1.00	1.00
95% CI	(61.6; 66.44)	(4.15; 4.93)	(61.27; 66.77)	(71.58; 79.03)	(5.92; 6.24)	(1.17; 1.48)	(1.68; 1.99)
Min	26	-2.8	16.1	24.0	2.9	-1.1	-2.3
Max	94	15.5	110.3	123.6	9.6	4.8	5.0
Median	64	4.4	63.0	76.2	6.1	1.2	1.9
Q1	53	2.9	51.9	57.5	5.3	0.7	1.2
Q3	75	5.8	74.3	90.0	6.6	1.8	2.3

Legend:
IA, integrated albumin; KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation; U, units.
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.

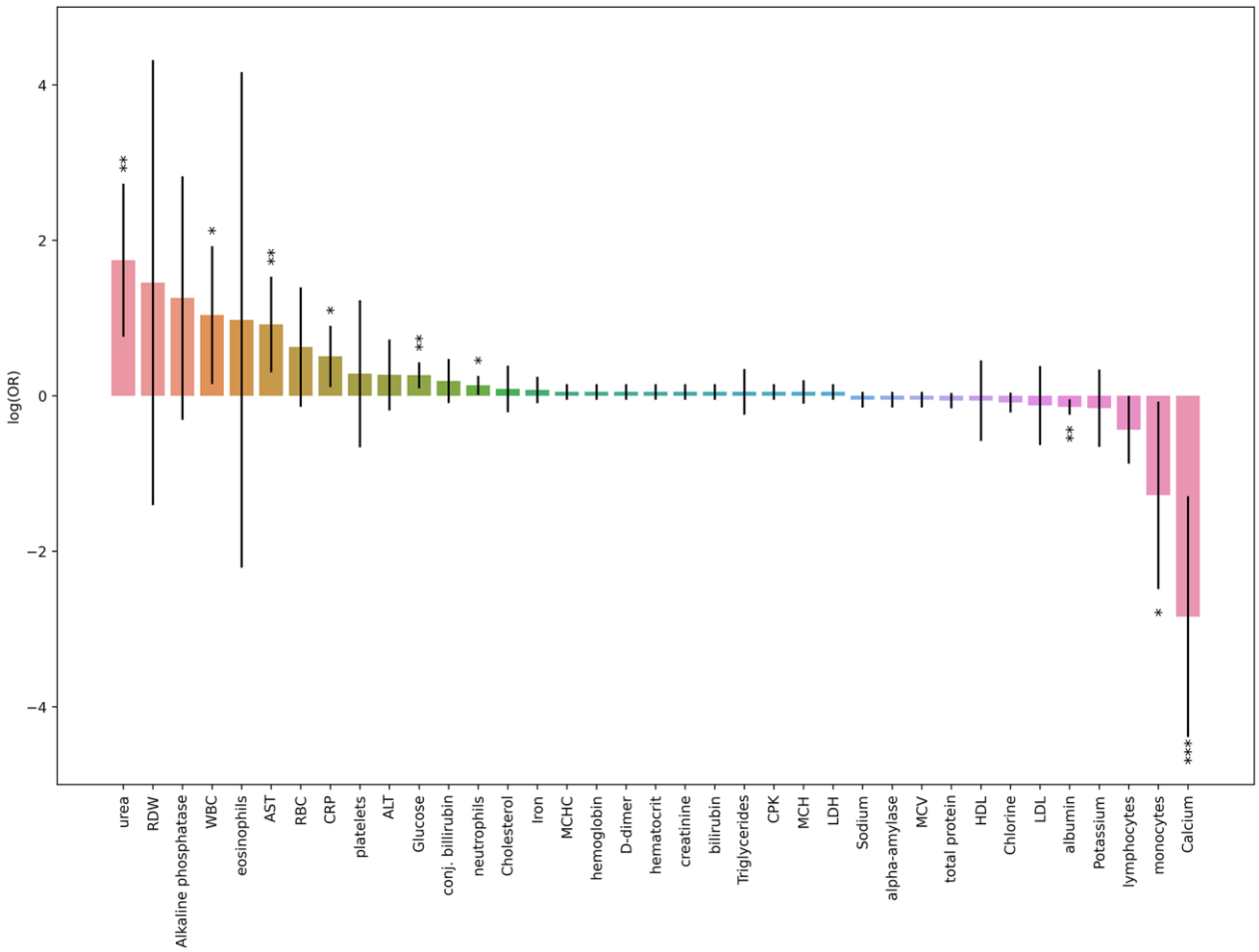

Figure 1. Results obtained from three-factor logistic regression models for blood tests results parameters and death risk. Height of each bar depicts log(OR) obtained from logistic regression model (age and sex was taken as covariates), black lines depicts 95% CI for each result. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 (the last one is suitable for Bonferroni adjustment).

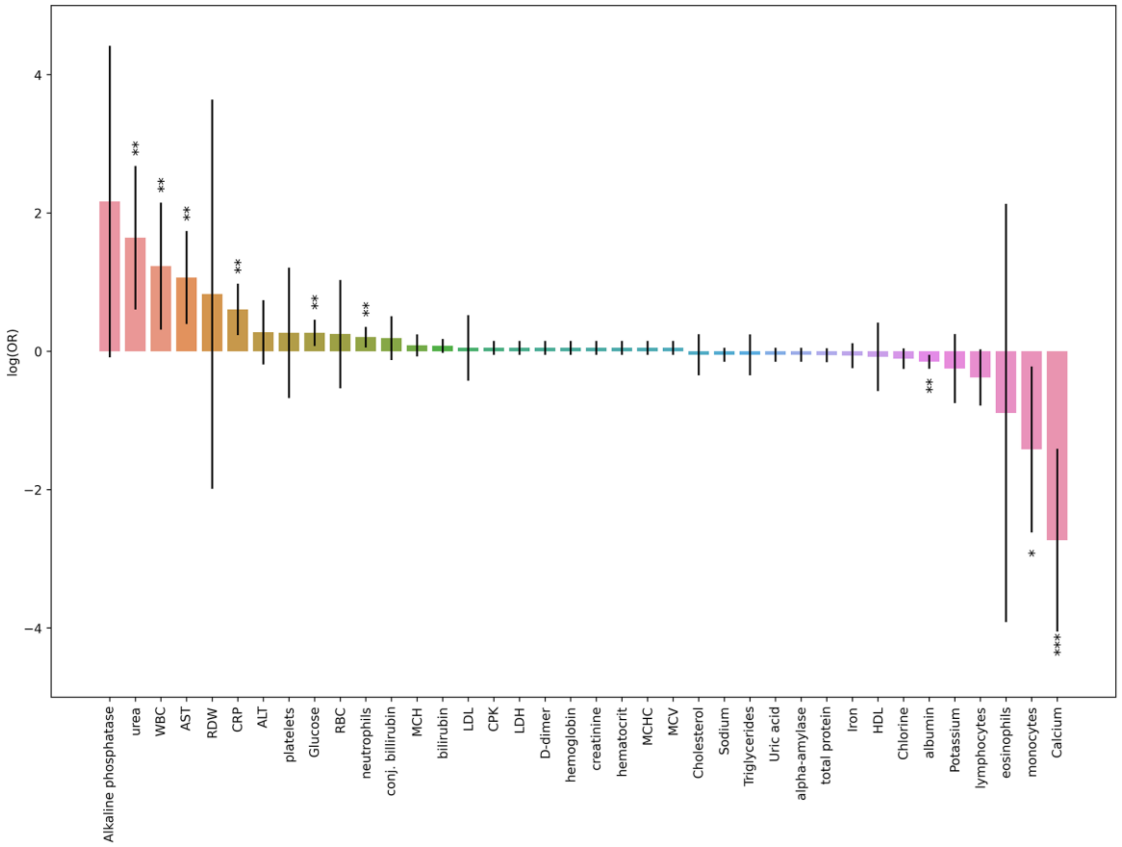

Figure 2. Results obtained from three-factor logistic regression models for blood tests results parameters and deterioration risk. Height of each bar depicts log(OR) obtained from logistic regression model (age and sex was taken as covariates), black lines depicts 95% CI for each result. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 (the last one is suitable for Bonferroni adjustment).

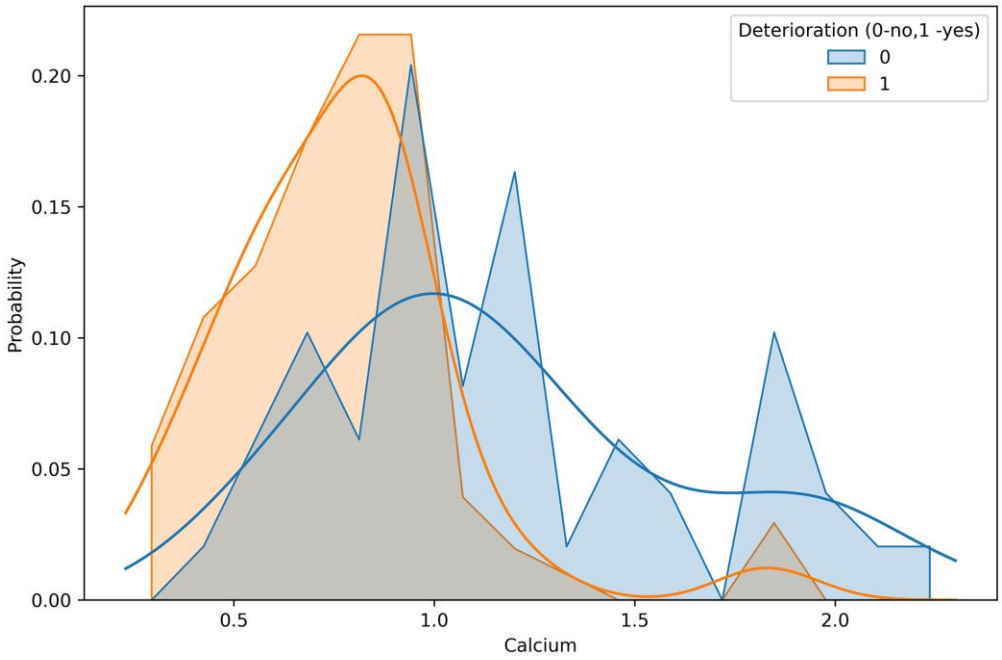

Figure 3. Calcium concentration distributions in groups differed by deterioration outcome.

Table 3. Death OR obtained by multivariate logistic regression.

Calculator	Factor	OR	p	95% CI for OR	
PD (g) for 0.5 units		1.683	<0.001	1.348	2.101
	Sex (female = ref)	2.553	0.039	1.050	6.209
	Age, for 5 years	1.604	<0.001	1.328	1.937
PD (o)		1.285	0.007	1.069	1.544
	Sex (female = ref)	2.885	0.014	1.237	6.731
	Age, for 5 years	1.575	<0.001	1.313	1.890
PD (l), 0,5 units		1.619	<0.001	1.247	2.101
	Sex (female = ref)	2.378	0.048	1.007	5.617
	Age, for 5 years	1.571	<0.001	1.307	1.887
KD, 5 units		1.449	<0.001	1.177	1.783
	Sex (female = ref)	4.370	0.065	0.915	20.870
	Age, for 5 years	1.147	0.429	0.817	1.609
PA, 5 units		1.623	<0.001	1.247	2.114
	Sex (female = ref)	2.936	0.093	0.835	10.328
	Age, for 5 years	1.079	0.608	0.808	1.440

Legend:
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.

per 5-unit increase in the KD age. Sex was not significantly associated with the outcome, while chronological age was significant only in the KD model.

We also checked whether combining these two scales would yield better results. To this end, we built four-factor models including all pairwise combinations of the global PD, PA, and KD for all three outcomes (Tables 6, 7). In all cases, only one of the two metrics showed a significant association with the outcome, whereas the second metric showed no independent contribution. For death, PA and KD remained significant, while global PD was not, and for deterioration, it was global PD that remained significant, while KD and PA did not.

Thus, we can say that some scales, especially the final 14 biomarker sets used for calculating the global PD, could serve as predictors for both deterioration and death in patients with COVID-19.

DISCUSSION

Recent studies showed that the severity of COVID-19 was more strongly associated with the biological age rather than the chronological age [14, 15]. In this study, we evaluated the possibility of using physiological state indices to predict disease outcomes. The hypothesis of this study was that summary metrics of physiological state, which take into account morphological, physiological, and functional characteristics of the organism, should better predict disease outcomes. According to our results, some physiological indices predicted a higher risk of mortality and deterioration in the models adjusted for chronological age. The global PD, calculated using the Mahalanobis distance (MD) [11] and including 14 biomarkers (mean corpuscular hemoglobin [MCH], red cell distribution width [RDW], red blood cell [RBC], platelets, percentage of lymphocytes, white blood cell [WBC], C-reactive protein [CRP], potassium, sodium, hemoglobin, albumin, alanine transaminase [ALT], aspartate aminotransferase [AST], and total protein), appeared to be one of the best predictors for death and deterioration of patients with COVID-19. Such results were expected because this calculator consists of crucial parameters for the outcomes. Therefore, WBC, CRP, and other biomarkers, which are commonly used in clinical practice to evaluate COVID-19 severity, along with the chronological age, can be combined into integral models to determine the risk of unfavorable outcomes of the disease. However, the MD calculation involves a

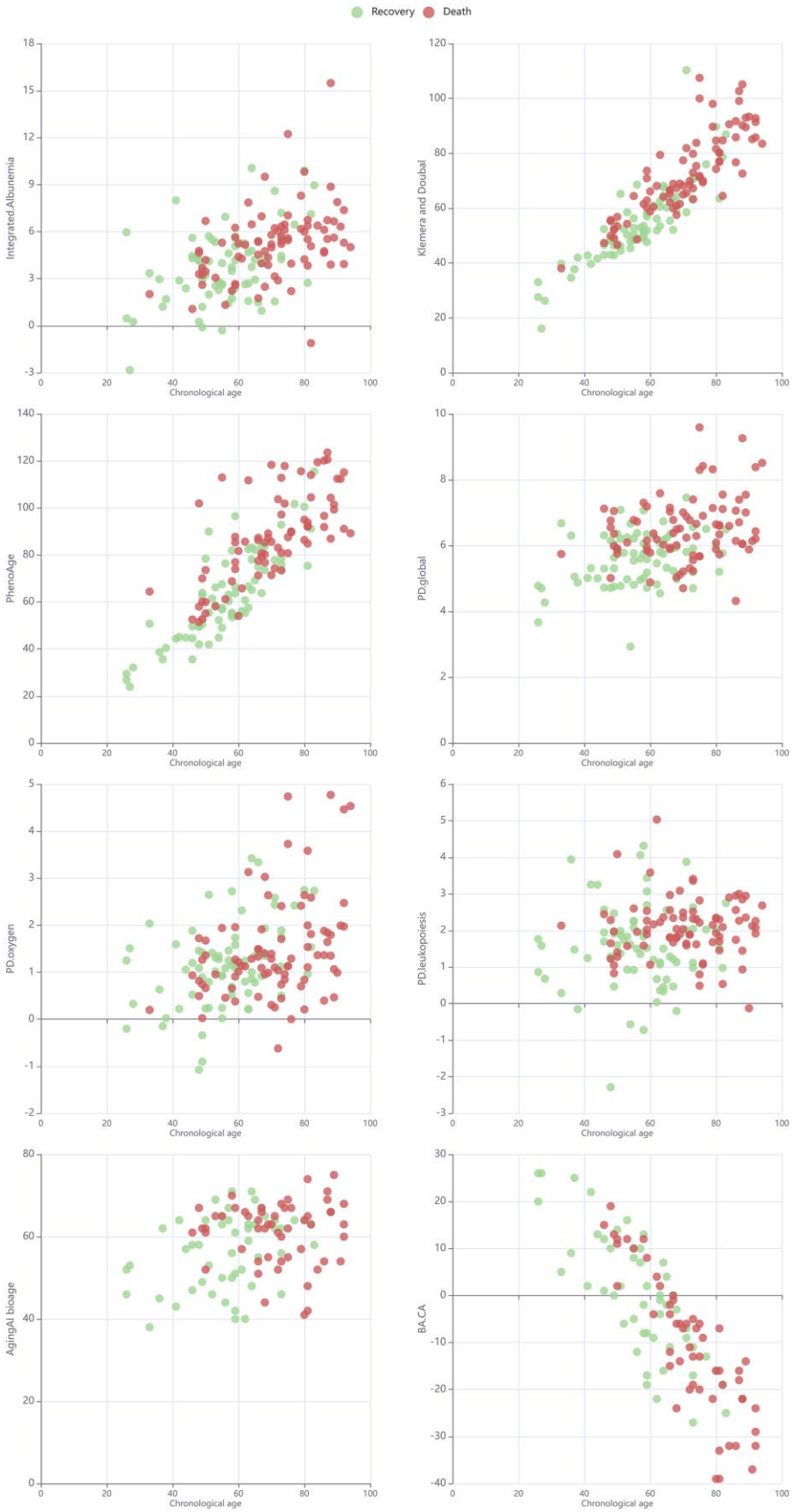

Figure 4. Scatter plot for chronological age and selected metrics of biological age or physiological state in cohorts split by death/recovery.

Table 4. COVID-19 course deterioration OR obtained by multivariate logistic regression.

Calculator	Factor	OR	p	95%CI for OR	
PD (g) for 0.5 units		1.417	<0.001	1.271	1.580
	Sex (female = ref)	1.113	0.593	0.752	1647
	Age, for 5 years	0.988	0.706	0.926	1.05
PD (o)		1.017	0.728	0.927	1.115
	Sex (female = ref)	1.327	0.138	0.913	1.929
	Age, for 5 years	1.031	0.324	0.970	1.097
PD (l), 0,5 units		1.329	<0.001	1.194	1.480
	Sex (female = ref)	1.396	0088	0.951	2.049
	Age, for 5 years	1.011	0.732	0.950	1.076
KD, 5 units		1.204	0.002	1.068	1.358
	Sex (female = ref)	1.583	0.132	0.871	2.877
	Age, for 5 years	0.819	0.010	0.705	0.953
PA, 5 units		1.131	0.116	0.970	1.319
	Sex (female = ref)	2.168	0.021	1.121	4.194
	Age, for 5 years	0.903	0.308	0.742	1.099

Legend:
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.

non-monotonic manipulation of each component variable and, as such, is not necessarily associated directly with higher levels of individual markers. It is important to note that, unlike other risk scores for COVID-19 severity, this index did not include assessment of comorbidities, for which an assessment could be complicated, especially in case of emergency hospitalizations. Interestingly, integrated albunemia had no association with COVID-19 outcomes, although some of its indicators, including the calcium levels, were strongly correlated with mortality and deterioration. In addition, it should be noted that in the combined indices in the same models, PD did not predict mortality anymore but was still an extremely strong predictor of deterioration. Therefore, we can say that the indices did not measure exactly the same thing.

The aging process is manifested in progressive systemic remodeling of body functioning; therefore, a number of biological dimensions are associated with this process. Most biological indices for age are associated with chronic diseases and unhealthy lifestyle. Strong associations between severe COVID-19 and biological age once again emphasize the importance of preventing aging, both in individuals and in the entire population. The strong association of PD with severe COVID-19 outcomes also suggests the importance of maintaining physiological equilibrium, regardless of age. Unlike PA and KD, the effect of chronological age remained strong in models with PD, suggesting that PD measures information that is more weakly associated with aging and yet is nonetheless critical for health.

Thus, our results are partly attributed to common chronic diseases, which aggravate COVID-19, but also suggest that biological age indices could capture vulnerability to severe COVID-19 and serve as a tool for course prediction and determination of tactics for patient management.

The biological age, as measured by different indices, was associated with a higher risk of mortality and deterioration in the models for which the chronological age and sex were adjusted. Thus, multivariate indices of the physiological state, including the PD, can be used to determine the risk of deterioration and death in a patient. PD measured using the MD could serve as a panel to assess patient risk because it is composed of common markers widely used in clinical practice.

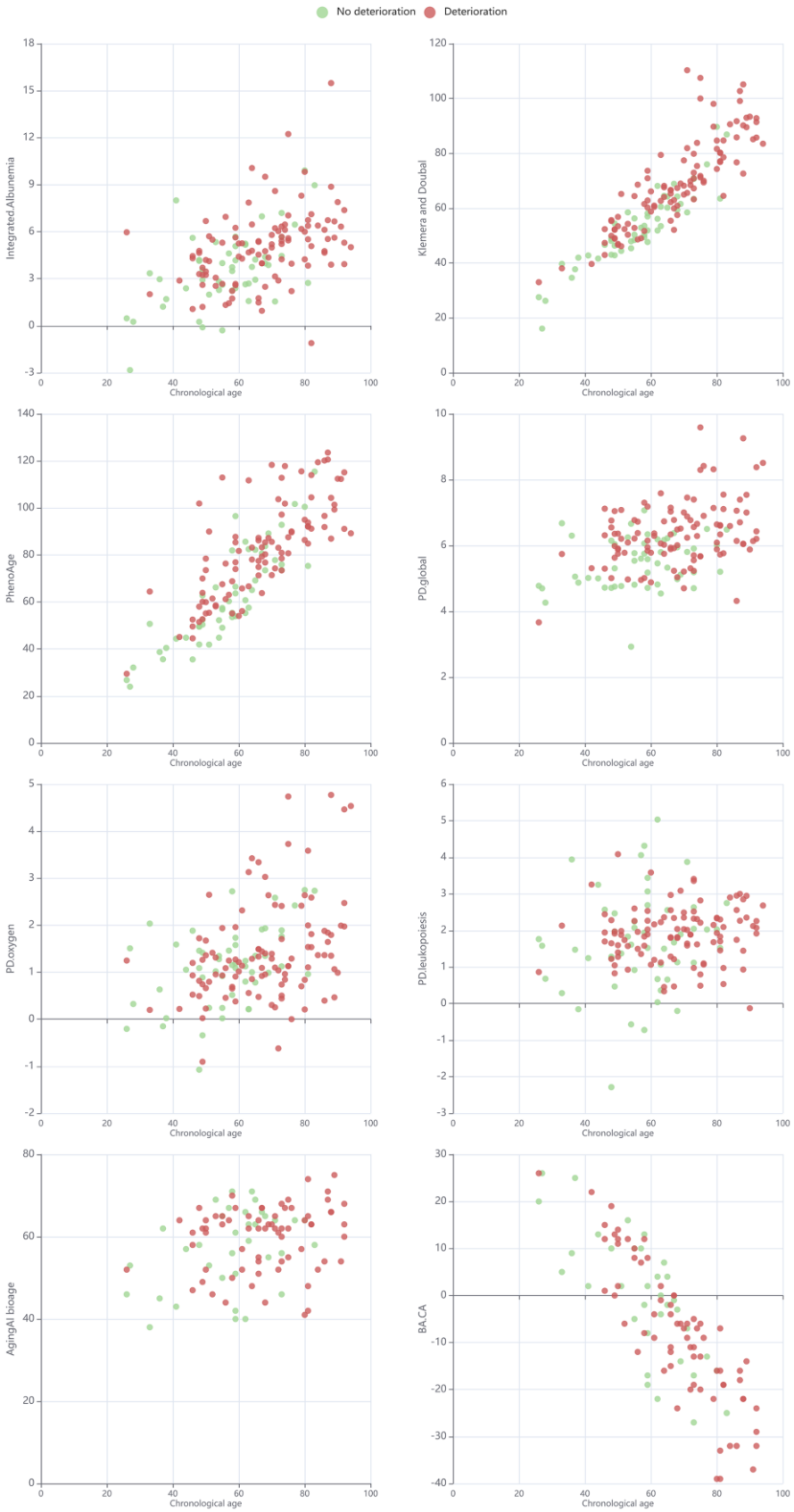

Figure 5. Scatterplot for chronological age and selected metrics of biological age or physiological state in cohorts split by deterioration.

Table 5. OR for the combined endpoint (death or deterioration of the patient's condition) obtained by multivariate logistic regression.

Calculator	Factor	OR	p	95% CI for OR	
PD (g) for 0.5 units		1.417	<0.001	1.271	1.580
	Sex (female = ref)	1.113	0.593	0.752	1.647
	Age, for 5 years	0.988	0.706	0.926	1.054
PD (l), 0,5 units		1.329	<0.001	1.194	1.480
	Sex (female = ref)	1.396	0.088	0.951	2.049
	Age, for 5 years	1.011	0.732	0.950	1.076
KD, 5 units		1.204	0.002	1.068	1.358
	Sex (female = ref)	1.583	0.132	0.871	2.877
	Age, for 5 years	0.819	0.010	0.705	0.953

Legend:
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.

Table 6. OR of death obtained by multivariate logistic regression.

Calculator	Factor	OR	p	95% CI for OR	
	Sex (female = ref)	2.276	0.217	0.616	8.404
	Age, for 5 years	1.069	0.668	0.789	1.448
PD (g) for 0.5 units		1.310	0.160	0.899	1.911
PhenoAge, 5 units		1.541	0.002	1.173	2.024
	Sex (female = ref)	3.662	0.125	0.697	19.233
	Age, for 5 years	1.190	0.330	0.838	1.690
PD (g) for 0.5 units		1.352	0.196	0.856	2.138
KD, 5 units		1.324	0.017	1.051	1.667
	Sex (female = ref)	2.907	0.277	0.424	19.940
	Age, for 5 years	1.097	0.684	0.702	1.715
PhenoAge, 5 units		1.146	0.620	0.668	1.967
KD, 5 units		1.368	0.092	0.950	1.971

Legend:
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.

Table 7. Odds ratios (OR) of patient deterioration obtained by multivariate logistic regression.

Calculator	Factor	OR	p	95% CI for OR	
	Sex (female = ref)	1.687	0.140	0.843	3.377
	Age, for 5 years	0.966	0.747	0.784	1.190
PD (g) for 0.5 units		1.479	0.001	1.173	1.864
PhenoAge, 5 units		1.012	0.891	0.854	1.200
	Sex (female = ref)	1.011	0.971	0.516	1.984
	Age, for 5 years	0.855	0.049	0.732	0.999

PD (g) for 0.5 units		1.592	<0.001	1.280	1.980
KD, 5 units		1.095	0.154	0.966	1.241
	Sex (female = ref)	3.093	0.020	1.194	8.016
	Age, for 5 years	0650	0.009	0.471	0.898
PhenoAge, 5 units		1359	0.061	0.986	1.874
KD, 5 units		1122	0.304	0.901	1.398

Legend:
KD, Klemera and Doubal biological age; PA, PhenoAge; PD, physiological dysregulation.
g: biomarker part of the final 14 biomarkers set used for global physiological dysregulation.
l: biomarker used for physiological dysregulation in the leukopoiesis system.
o: biomarker used for physiological dysregulation in the oxygen transport system.

AUTHOR CONTRIBUTIONS

All authors contributed equally.

CONFLICTS OF INTEREST

A. A. C. is the CEO and founder of Oken Health.

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REFERENCES

1. Mueller AL, McNamara MS, Sinclair DA. Why does COVID-19 disproportionately affect older people? Aging (Albany NY). 2020; 12:9959–81. https://doi.org/10.18632/aging.103344 PMID:32470948

2. Zhavoronkov A. Geroprotective and senoremediative strategies to reduce the comorbidity, infection rates, severity, and lethality in gerophilic and gerolavic infections. Aging (Albany NY). 2020; 12:6492–510. https://doi.org/10.18632/aging.102988 PMID:32229705

3. Cunha LL, Perazzio SF, Azzi J, Cravedi P, Riella LV. Remodeling of the immune response with aging: immunosenescence and its potential impact on COVID-19 immune response. Front Immunol. 2020; 11:1748. https://doi.org/10.3389/fimmu.2020.01748 PMID:32849623

4. Pranata R, Henrina J, Lim MA, Lawrensia S, Yonas E, Vania R, Huang I, Lukito AA, Suastika K, Kuswardhani RA, Setiati S. Clinical frailty scale and mortality in COVID-19: A systematic review and dose-response meta-analysis. Arch Gerontol Geriatr. 2021; 93:104324. https://doi.org/10.1016/j.archger.2020.104324 PMID:33352430

5. Levine ME. Modeling the rate of senescence: can estimated biological age predict mortality more accurately than chronological age? J Gerontol A Biol Sci Med Sci. 2013; 68:667–74. https://doi.org/10.1093/gerona/gls233 PMID:23213031

6. Kuo CL, Pilling LC, Atkins JL, Masoli JA, Delgado J, Tignanelli C, Kuchel GA, Melzer D, Beckman KB, Levine ME. Biological aging predicts vulnerability to COVID-19 severity in UK Biobank participants. J Gerontol A Biol Sci Med Sci. 2021; 76:e133–41. https://doi.org/10.1093/gerona/glab060 PMID:33684206

7. Cohen AA, Milot E, Li Q, Bergeron P, Poirier R, Dusseault-Bélanger F, Fülöp T, Leroux M, Legault V, Metter EJ, Fried LP, Ferrucci L. Detection of a novel, integrative aging process suggests complex physiological integration. PLoS One. 2015; 10:e0116489. https://doi.org/10.1371/journal.pone.0116489 PMID:25761112

8. Klemera P, Doubal S. A new approach to the concept and computation of biological age. Mech Ageing Dev. 2006; 127:240–8. https://doi.org/10.1016/j.mad.2005.10.004 PMID:16318865

9. Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, Hou L, Baccarelli AA, Stewart JD, Li Y, Whitsel EA, Wilson JG, Reiner AP, et al. An epigenetic biomarker of aging for lifespan and healthspan. Aging (Albany NY). 2018; 10:573–91.

https://doi.org/10.18632/aging.101414
PMID:29676998

10. Cohen AA, Milot E, Yong J, Seplaki CL, Fülöp T, Bandeen-Roche K, Fried LP. A novel statistical approach shows evidence for multi-system physiological dysregulation during aging. Mech Ageing Dev. 2013; 134:110–7. https://doi.org/10.1016/j.mad.2013.01.004 PMID:23376244

11. Cohen AA, Milot E, Li Q, Legault V, Fried LP, Ferrucci L. Cross-population validation of statistical distance as a measure of physiological dysregulation during aging. Exp Gerontol. 2014; 57:203–10. https://doi.org/10.1016/j.exger.2014.04.016 PMID:24802990

12. Cohen AA, Li Q, Milot E, Leroux M, Faucher S, Morissette-Thomas V, Legault V, Fried LP, Ferrucci L. Statistical distance as a measure of physiological dysregulation is largely robust to variation in its biomarker composition. PLoS One. 2015; 10:e0122541. https://doi.org/10.1371/journal.pone.0122541 PMID:25875923

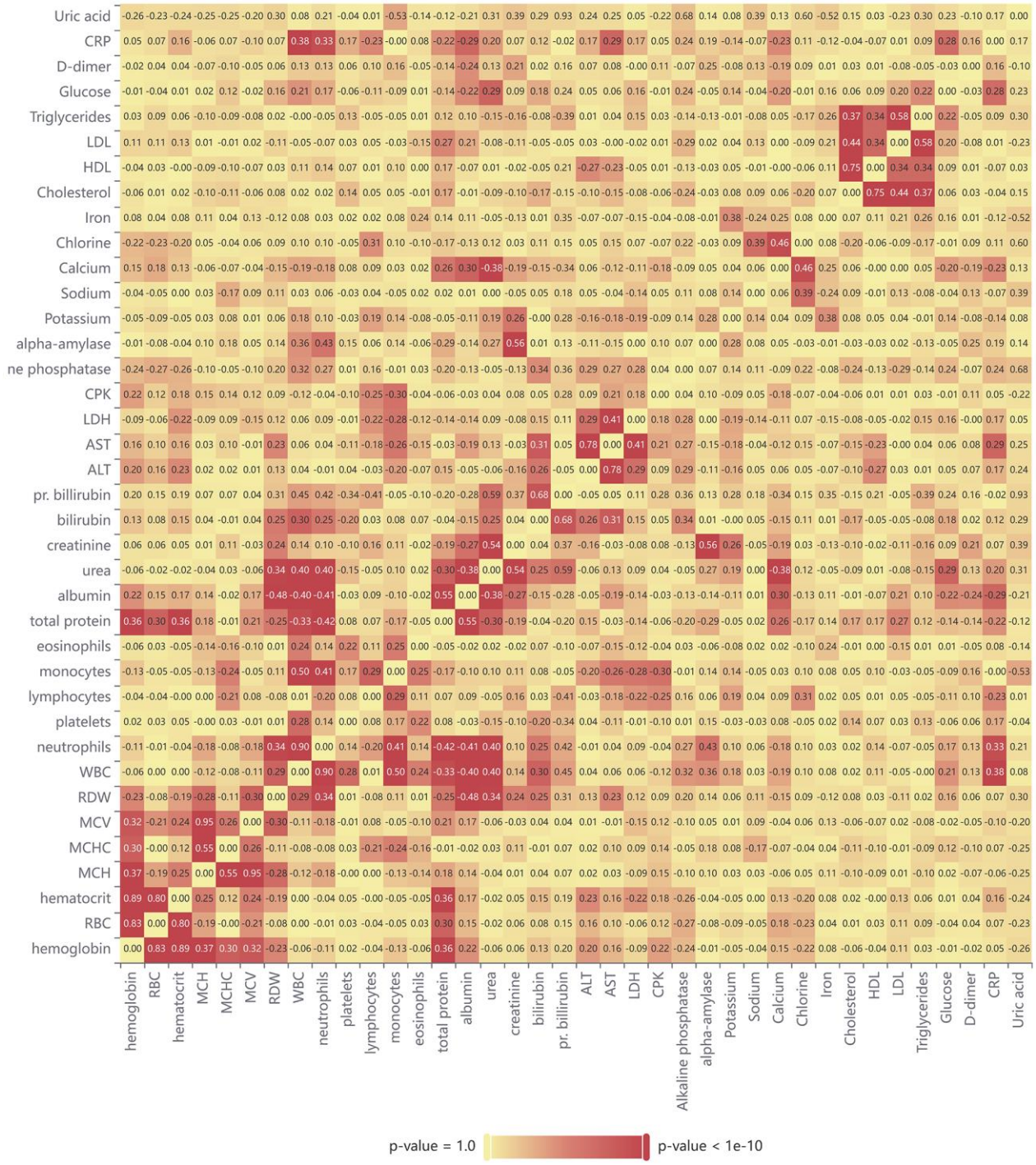
13. Li Q, Wang S, Milot E, Bergeron P, Ferrucci L, Fried LP, Cohen AA. Homeostatic dysregulation proceeds in parallel in multiple physiological systems. Aging Cell. 2015; 14:1103–12. https://doi.org/10.1111/acel.12402 PMID:26416593

14. Polidori MC, Sies H, Ferrucci L, Benzing T. COVID-19 mortality as a fingerprint of biological age. Ageing Res Rev. 2021; 67:101308. https://doi.org/10.1016/j.arr.2021.101308 PMID:33621704

15. Lauc G, Sinclair D. Biomarkers of biological age as predictors of COVID-19 disease severity. Aging (Albany NY). 2020; 12:6490–1. https://doi.org/10.18632/aging.103052 PMID:32268300

SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Correlation heatmap for CBC indicators, biochemical blood test biomarkers, and additional biomarkers. The color represents the significance of the observed correlation derived from the Pearson's correlation test.

Supplementary Tables

Supplementary Table 1. Descriptive statistics of the cell blood count indicators.

Parameter	Hemoglobin	RBC	Hematocrit	MCH	MCHC	MCV	RDW	WBC	Neutrophils	Platelets	Lymphocytes	Monocytes	Eosinophils
N	155	155	155	155	155	155	155	155	155	155	155	155	155
M	129.45	4.32	38.71	30.08	332.40	90.50	14.19	7.75	5.84	197.61	1.31	0.51	0.09
SD	17.67	0.55	5.89	2.62	9.80	6.83	3.36	4.41	4.22	80.23	0.88	0.30	0.11
95% CI	(126.64; 132.25)	(4.23; 4.40)	(37.77; 39.64)	(29.66; 30.50)	(330.84; 333.96)	(89.41; 91.58)	(13.66; 14.72)	(7.05; 8.45)	(5.17; 6.51)	(184.88; 210.34)	(1.17; 1.45)	(0.46; 0.55)	(0.07; 0.10)
Min	43	1.9	14.1	20.9	298.0	65.2	11.4	2.3	1.25	54	0.14	0.09	0
Max	180	5.5	53.5	41.8	362.0	124.4	47.5	28.3	25.2	525	6.9	2.18	0.8
Me	131	4.4	39.0	30.2	331.0	90.6	13.5	6.5	4.46	178	1.2	0.44	0.06
Q1	120	4.0	35.9	28.9	326.0	87.4	12.7	4.9	3.2	143	0.76	0.3	0.034
Q3	140	4.7	42.3	31.4	338.0	93.6	14.8	9.3	7.65	241	1.63	0.63	0.1

Supplementary Table 2. Descriptive statistics of the biochemical blood test indicators.

Parameter	Total protein	Albumin	Urea	Creatinine	Bilirubin	Probillirubin	ALT	AST	LDH	CPK	Alkaline phosphatase
N	155	155	155	155	155	29	154	154	137	124	63
M	63.99	33.76	8.00	120.94	13.00	9.59	49.18	67.08	852.91	316.94	223.27
SD	6.47	5.37	6.02	84.84	9.91	4.95	62.03	79.58	467.86	329.73	158.91
95% CI	(62.97; 65.02)	(32.91; 34.61)	(7.05; 8.96)	(107.48; 134.4)	(11.42; 14.57)	(7.71; 11.47)	(39.30; 59.05)	(54.42; 79.75)	(773.86; 931.96)	(258.33; 375.56)	(183.25; 263.29)
Min	45	18.8	2.1	37.0	4.0	3	3	10	231	12	74
Max	79	46.0	45.3	761.0	69.2	19.9	445	610	2591	1986	1136
Me	64.9	34.0	6.2	99.0	10.4	8.7	32	43	748	197.5	182
Q1	60	30.0	4.4	87.0	7.3	5.2	19	28	506	106.5	137
Q3	68	37.8	8.5	128.0	14.1	13.6	52	66	1039	378.5	241
Parameter	**Alphaamylase**	**Potassium**	**Sodium**	**Calcium**	**Chlorine**	**Iron**	**Cholesterol**	**HDL**	**LDL**	**Triglycerides**	**Glucose**
N	55	155	155	151	76	51	155	109	109	154	155
M	59.91	3.86	138.93	0.90	102.18	8.46	4.23	1.52	2.67	2.59	7.96
SD	50.05	0.73	5.12	0.40	5.74	5.69	1.25	0.88	0.89	1.24	3.60
95% CI	(46.38; 73.44)	(3.75; 3.98)	(138.12; 139.74)	(0.83; 0.96)	(100.87; 103.5)	(6.86; 10.06)	(4.03; 4.43)	(1.35; 1.68)	(2.50; 2.83)	(2.40; 2.79)	(7.38; 8.53)
Min	17	2.4	119	0.23	79	2.2	1.3	0.3	0.9	0.61	4.2
Max	222	6.3	160	2.3	121	26.4	7.8	4.5	5.8	6.9	23.6
Me	47	3.8	139	0.86	102	6.2	3.9	1.2	2.7	2.5	6.6
Q1	31	3.3	136	0.63	99	4.1	3.2	0.9	2.1	1.8	5.7
Q3	65	4.3	142	1	106	10.7	5	2	3.1	3.1	8.6

Supplementary Table 3. Descriptive statistics for additional markers.

Parameter	D-dimer	CRP	Uric acid
N	128	155	18
M	1527.77	117.43	262.67
SD	1319.41	88.63	104.66
95% CI	(1297.00; 1758.54)	(103.36; 131.49)	(210.62; 314.71)
Min	76	2	135
Max	4000	436	567
Me	904	102	254
Q1	430	51	194
Q3	2575	162	324

Supplementary Table 4. Descriptive statistics for categorical data.

Factor	Meaning	Number of patients (%)
Sex	Male	82/155 (52.9%)
	Female	73/155 (47.1%)
DM2	No	126/155 (81.3%)
	Yes	29/155 (18.7%)
CAD	No	69/155 (44.5%)
	Yes	86/155 (55.5%)
AH	No	51/155 (32.9%)
	Yes	104/155 (67.1%)
CHF	No	93/155 (60.0%)
	Yes	62/155 (40.0%)
Onco Anamnesis	No	143/155 (92.3%)
	Yes	12/155 (7.7%)
COPD	No	129/155 (832%)
	Yes	26/155 (16.8%)

Note: N, cohort size; M, mean, DM2, diabetes mellitus type 2; CAD, coronary artery disease; AH, arterial hypertension; CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; CBC, cell blood count.

Research Paper

Association of active immunotherapy with outcomes in cancer patients with COVID-19: a systematic review and meta-analysis

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ABSTRACT

Background: During the COVID-19 pandemic, there are growing concerns about the safety of administering immunotherapy in cancer patients with COVID-19. However, current clinical guidelines provided no clear recommendation.

Methods: Studies were searched and retrieved from electronic databases. The meta-analysis was performed by employing the generic inverse-variance method. A random-effects model was used to calculate the unadjusted odds ratios (ORs) and adjusted ORs with the corresponding 95% CIs.

Results: This meta-analysis included 20 articles with 6,042 cancer patients diagnosed with COVID-19. According to the univariate analysis, the acceptance of immunotherapy within 30 days before COVID-19 diagnosis did not increase the mortality of cancer patients (OR: 0.92; 95% CI: 0.68-1.25; *P*=0.61). Moreover, after adjusting for confounders, the adjusted OR for mortality was 0.51, with borderline significance (95% CI: 0.25-1.01; *P*=0.053). Similarly, the univariate analysis showed that the acceptance of immunotherapy within 30 days before COVID-19 diagnosis did not increase the risk of severe/critical disease in cancer patients (OR: 1.07; 95% CI: 0.78-1.47; *P*=0.66). No significant between-study heterogeneity was found in these analyses.

Conclusions: Accepting immunotherapy within 30 days before the diagnosis of COVID-19 was not significantly associated with a higher risk of mortality or severe/critical disease of infected cancer patients. Further prospectively designed studies with large sample sizes are required to evaluate the present results.

INTRODUCTION

As of 17 September 2021, a total of 226,844,344 confirmed COVID-19 cases were reported globally, and 4,666,334 COVID-19-related deaths were reported [1]. Cancer is revealed to be independently associated with the risk of COVID-19 and is significantly related to an increased rate of severe disease and mortality [2]. SARS-CoV-2 has been shown to trigger cytokine storms, leading to the increment of the incidence of acute respiratory distress syndrome (ARDS). Thus, during the pandemic, some oncologists have growing concerns about the safety of immunotherapy because of the risk of the uncontrolled inflammatory response of patients who are infected with COVID-19 [3]. Due to the unclear impacts of immunotherapy on cancer patients who had concurrent COVID-19, oncologists are currently confronted with difficulties in the management and

treatment of cancer patients [4]. Given this, the delay or cancellation of planned immunotherapy might occur, negatively affecting patients' prognosis [5].

Although most clinical trials of immunotherapy have precluded patients with active virus infection because of concerns about disease reactivation and immune-related adverse events [6], a few studies assessing the effects of implementing immunotherapy in cancer patients with active virus infection have shown that different viruses have different effects on prognosis. Regarding human immunodeficiency virus (HIV), a prior study indicated that immunotherapy was safe and feasible among patients with non-small-cell lung cancer (NSCLC) and active HIV infection, and the expression of programmed death-ligand 1 (PD-L1) was much higher in infected individuals than in their counterparts [7]. Moreover, in terms of human papillomavirus (HPV), the results of a phase 3 clinical trial focusing on unresectable or metastatic head and neck cancer showed that immunotherapy had more benefit on overall survival (OS) among PD-L1 expressors with HPV-positive tumours than their counterparts with HPV-negative tumours [8]. Similarly, Epstein–Barr virus (EBV) infection was found to be significantly associated with positive programmed death-1 (PD-1) staining in patients with locoregionally advanced nasopharyngeal carcinoma, and positive PD-1 staining was independently identified as a predictor for improved prognosis [9]. However, with regard to hepatitis B virus (HBV), a clinical trial in patients with B-cell lymphoma illustrated the risk of HBV reactivation when patients coinfected with HBV were receiving immunotherapy, which might lead to a poorer prognosis [10]. Additionally, Tapia Rico et al. [11] indicated that HBV-positive cancer patients who were treated with ICIs had the hazard of developing HBV reactivation, which could result in severe or critical immune-related adverse events.

For SARS-CoV-2, many studies have been published, while only a marginal number of prior meta-analyses have focused on the efficiency and safety of administering immunotherapy for cancer patients during the pandemic, and solid evidence is still lacking [12, 13]. Because of the lack of evidence, current clinical practice guidelines provide no clear recommendation for administering immunotherapy in cancer patients who had concurrent COVID-19. The World Health Organization (WHO) recommended continuing or modifying previous treatment according to the patient's clinical condition [14]. Similarly, the American Society of Clinical Oncology (ASCO) and the European Society for Medical Oncology (ESMO) recommended making decisions on the basis of discreet evaluation of benefits and risks for cancer patients [4, 15].

Hence, considering the points mentioned above, we conducted the present meta-analysis, for evaluating the relationship between active immunotherapy and outcomes of cancer patients who had concurrent COVID-19 infection.

MATERIALS AND METHODS

We conducted and reported the systematic review and meta-analysis, in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [16]. The protocol of the systematic review and meta-analysis has been registered in the PROSPERO database (CRD42021274069).

Inclusion and exclusion criteria

The eligibility of the studies was assessed according to the following criteria. (1) The participants included cancer patients who were diagnosed with COVID-19 by RT–PCR and receiving active immunotherapy. (2) The intervention included immunotherapy within 30 days before the diagnosis of COVID-19. (3) The control group included cancer patients who did not receive immunotherapy within 30 days before the diagnosis of COVID-19. (4) The primary outcome was defined as mortality, and the secondary outcome was defined as the rate of severe/critical disease. The definition of severe/critical disease was in accordance with the WHO guidelines [14]. (5) All kinds of prospective and retrospective studies with extractable odds ratios (ORs), relative risks (RRs), or relevant statistics to calculate ORs and RRs were included. If the same cases from the same cohort were reported in more than one study, only the most recent study or the study reporting the most cases was included.

Studies were excluded based on the following exclusion criteria: (1) basic research, review, news, conference, guideline, editorial, comment, clinical experience, case report, and study protocol; (2) studies in which data were missing from a group of patients or data of patients receiving immunotherapy could not be separated from the whole patient group; (3) patients in whom cancer had been cured before the diagnosis of COVID-19; and (4) patients who were diagnosed with other viral pneumonias.

Search strategy

A comprehensive search strategy was designed and performed. Studies were searched and retrieved from databases, including Embase, the Cochrane Library, Web of Science, PubMed, and the China National Knowledge Infrastructure (CNKI). The published

studies were searched from 01-Dec-2019 to 01-Aug-2021. Supplementary Table 1 presents the details of the search strategy for different databases. No language limitations were imposed. Moreover, the reference lists of the studies were reviewed, to search for relevant articles.

Two independent reviewers blinded to each other performed the screening process. The titles and abstracts of retrieved records were initially screened for eligibility. Following this, the full-text screening was performed to obtain eligible studies, in accordance with the inclusion and exclusion criteria. If any discrepancy between the reviewers emerged, it was solved by discussion and arbitration.

Data extraction

Data were extracted and collected from the included studies by two independent reviewers. Any discrepancy between the reviewers was solved by discussion and arbitration. The following datasets were extracted and collected by using a worksheet: name of the first author, publication year, country, study types (prospective or retrospective studies), total figure for participants, figure for males and females, median age or mean age, cancer types, number of patients receiving active immunotherapy, immunotherapy interval before COVID-19 diagnosis, and outcomes. The unadjusted and adjusted ORs were obtained from the articles. For adjusted ORs, adjusting variables for multivariate analyses were also extracted. If ORs were not provided, they were calculated based on original statistics, or RRs were extracted instead.

Quality assessment

The Newcastle–Ottawa Quality Assessment Scale (NOS) was employed to conduct the quality assessment. The scale for cohort studies was defined as 3 sections, encompassing selection (4 points), comparability (2 points), and outcome (3 points). Similarly, the scale for case-control studies was defined as 3 sections, encompassing selection (4 points), comparability (2 points), and exposure (3 points). A study was rated as low quality if it scored less than 5 points [17]. Two independent reviewers, blinded to each other, evaluated the risk of bias. If any disagreements emerged in the process, they were discussed or settled by the third reviewer.

Statistical analysis

The meta-analysis was performed by employing the generic inverse-variance method. ORs and their 95% confidence intervals (95% CIs) were calculated to compare the mortality and severe/critical disease rate between patients receiving active immunotherapy and the control patients. $P<0.05$ was considered statistically significant. As for heterogeneity analysis, the Cochran's Q test and inconsistency index (I^2) were adopted, with $I^2>50\%$ or $P<0.1$ deemed to indicate significant heterogeneity. We used random-effects model in the calculation of the pooled ORs and corresponding 95% CIs. Moreover, we conducted subgroup analyses according to study type, number of patients, cancer type, immunotherapy interval prior to the COVID-19 diagnosis, and number of patients receiving active immunotherapy.

We performed Egger's linear regression tests and Begg's rank correlation tests to estimate publication bias, in which $P<0.1$ indicated significant publication bias. Moreover, funnel plots were also provided to demonstrate the publication bias.

Furthermore, a sensitivity analysis was also performed by excluding an individual study each time to reflect whether any single study influenced the results.

A meta-regression was performed to investigate the effects of any potential sources of heterogeneity (study type, number of patients, number of patients receiving active immunotherapy, cancer type, and interval between immunotherapy and diagnosis of COVID-19). $P<0.1$ was considered statistically significant. The permutation test was also performed to validate the robustness of meta-regression. The data synthesis was performed by using RevMan, version 5.4 (The Nordic Cochrane Center, The Cochrane Collaboration, Denmark). Publication bias was calculated by using Stata, version 13.1 (Stata Corp, College Station, USA). Meta-regression and permutation tests were performed using the metafor package in R, version 5.1.1 (The R Foundation for Statistical Computing).

Data availability statement

Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online Supplementary Information.

RESULTS

Study selection

Figure 1 displays the flow diagram of study selection process. Overall, 6,236 articles were initially retrieved from the electronic databases, including 2,895 articles from EMBASE, 202 articles from the Cochrane Library, 1,728 articles from PubMed, 1,258 articles from Web of Science, and 153 articles from CNKI.

Then, 1,064 duplicate studies were excluded, followed by the screening process. Fifty-four potentially eligible articles remained and were assessed for eligibility by full-text screening. Finally, 20 articles fully meeting the inclusion criteria were included in subsequent meta-analysis [18–37]. Supplementary Table 2 lists the articles assessed for eligibility by full-text screening, in which reasons why studies were excluded are also shown.

Study main characteristics

Table 1 demonstrates the baseline characteristics of the included studies. Overall, five of the 20 included studies were prospective cohort studies [22, 29, 30, 35, 37]. The remainder were retrospectively designed [18–21, 23–28, 31–34, 36]. As a whole, 6,042 cancer patients diagnosed with COVID-19 were included, with 464 patients receiving active immunotherapy. Among the included 20 studies, five of them included patients with solid cancer [20, 23, 29, 33, 35], and four studies included patients with haematological malignancies [19, 30, 32, 37]. With regard to the remaining 11 studies, the included cancer types were nonspecific [18, 21, 22, 24–28, 31, 34, 36].

Supplementary Table 3 mirrors the results of the quality assessment. The NOS score of the included articles ranged between 6 and 8. None of the included studies was rated as low quality.

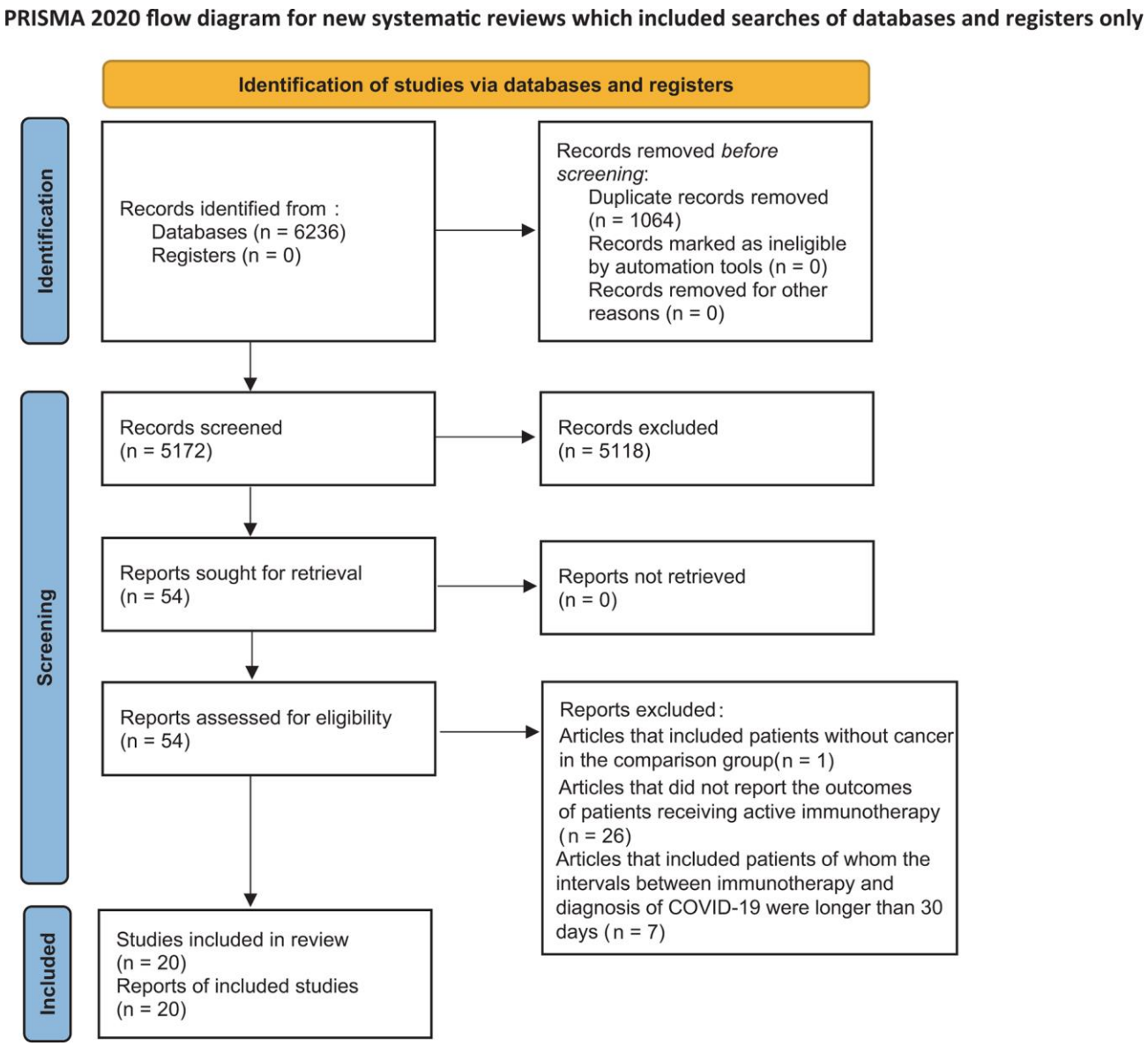

Figure 1. PRISMA flow diagram of study inclusion.

Table 1. Characteristics of the included studies.

Study ID	Study type	Country	Number of patients	M/F[a]	Median age (IQR)(years)[b]	Cancer type	Interval[c](days)	Number of patients[d]
Assaad 2020	Retrospective	France	302	144/158	58.2[#]	Non-specific	30	26
Fox 2020	Retrospective	UK	55	38/17	63(23-88)	Hematological malignancies	14	9
Garassino 2020	Retrospective	International	200	141/59	68(61.8-75)	Thoracic cancer	7 (median)	34
García-Suárez 2020	Prospective	Spain	697[e]	413/277	72 (60-79)	Hematological malignancies	30	44
Jee 2020	Retrospective	US	309	119/150	NA[f]	Non-specific	35	18
Lee 2020	Prospective	UK	800[g]	449/349	69(59-76)	Non-specific	28	44
Lievre 2020	Retrospective	France	1289	795/494	67(19-100)	Solid cancer	28	62
Mehta 2020	Retrospective	US	218	127/91	69(10-92)	Non-specific	30	5
Mehta 2021	Retrospective	India	186	105/81	52(42–58.75)	Non-specific	30	11
Nakamura 2021	Retrospective	Japan	32	22/10	74.5(24–90)	Non-specific	30	3
Ozer 2021	Retrospective	US	68	37/31	72(23-91)	Non-specific	28	2
Pinato 2020	Retrospective	International	890	503/387	68[#]	Non-specific	19 (mean)	56
Provencio 2021	Prospective	Spain	447	332/115	67.1[#]	Lung cancer	NA	91
Sanchez-Pina 2020	Prospective	Spain	39	23/16	64.7[#]	Hematological malignancies	NA	3
Stroppa 2020	Retrospective	Italy	25	20/5	71.64[#]	Non-specific	NA	4
Wang 2020	Retrospective	US	58	30/28	67	Multiple myeloma	NA	32
Yang F 2020	Retrospective	China	52	28/24	63(34–98)	Solid cancer	30	1
Yang KY 2020	Retrospective	China	205	96/109	63(56–70)	Non-specific	28	4
Yarza 2020	Prospective	Spain	63	34/29	66[#]	Solid cancer	28	8
Zhang 2020	Retrospective	China	107	60/47	66(36-98)	Non-specific	30	6

[a]M means males and F means females.
[b]IQR means interquartile range.
[c]Interval of immunotherapy before diagnosis of COVID-19.
[d]Number of cancer patients receiving immunotherapy within 30 days before COVID-19 diagnosis.
[e]Data are missing for 7 patients.
[f]NA means data not available.
[g]2 patients did not identify as either male or female.
[#]Mean age.

The effects of immunotherapy on cancer patients with COVID-19

According to the univariate analysis, the acceptance of active immunotherapy was not in relation to the increased mortality of cancer patients (OR: 0.92; 95% CI: 0.68-1.25; P=0.61), with no significant between-study heterogeneity found (I^2=4%; P=0.41). A forest plot of the unadjusted OR for the relationship between active immunotherapy and mortality of cancer patients who had concurrent COVID-19 is shown in Figure 2. Moreover, after adjusting for confounders, the adjusted OR was 0.51, with borderline significance (95% CI: 0.25-1.01; P=0.053), as shown in Figure 3. No significant heterogeneity was observed between the studies providing adjusted results of mortality (I^2=0%; P=0.55).

Subgroup analyses for mortality of cancer patients co-diagnosed with COVID-19 were performed, of which the unadjusted ORs are mirrored in Table 2. As reflected in the subgroup analyses, no variables investigated were related to significant ORs (P>0.05), revealing that active immunotherapy was not in relation to increased mortality of cancer patients, regardless of confounders.

For the meta-analysis of the severe/critical disease rate, the definition of WHO guidelines was employed [14].

Figure 4 shows a forest plot of the OR for the relationship between active immunotherapy and the rate of severe/critical disease in cancer patients who had concurrent COVID-19. According to the univariate analysis, active immunotherapy was not in relation to increased risk of severe/critical disease of cancer patients (OR: 1.07; 95% CI: 0.78-1.47; P=0.66), with nonsignificant between-study heterogeneity found (I^2=0%; P=0.92).

Supplementary Tables 4, 5 mirrors the results of meta-regression. None of the tested covariates could yield heterogeneity, with P>0.1. Permutation tests showed that the results of meta-regression were reliable.

Sensitivity analysis and publication bias

In order to evaluate the stability of the present results, sensitivity analyses were performed by excluding an individual study each time to reflect whether any single study influenced the results. The results of the sensitivity analysis indicated that the pooled ORs were not significantly influenced by excluding any single study. Furthermore, Egger's linear regression tests and Begg's rank correlation tests were conducted through which nonsignificant publication bias was shown in the studies regarding mortality and the studies regarding severe/critical disease, as illuminated in Supplementary Figures 1–4. Supplementary Figures 5, 6 are funnel plots of included studies.

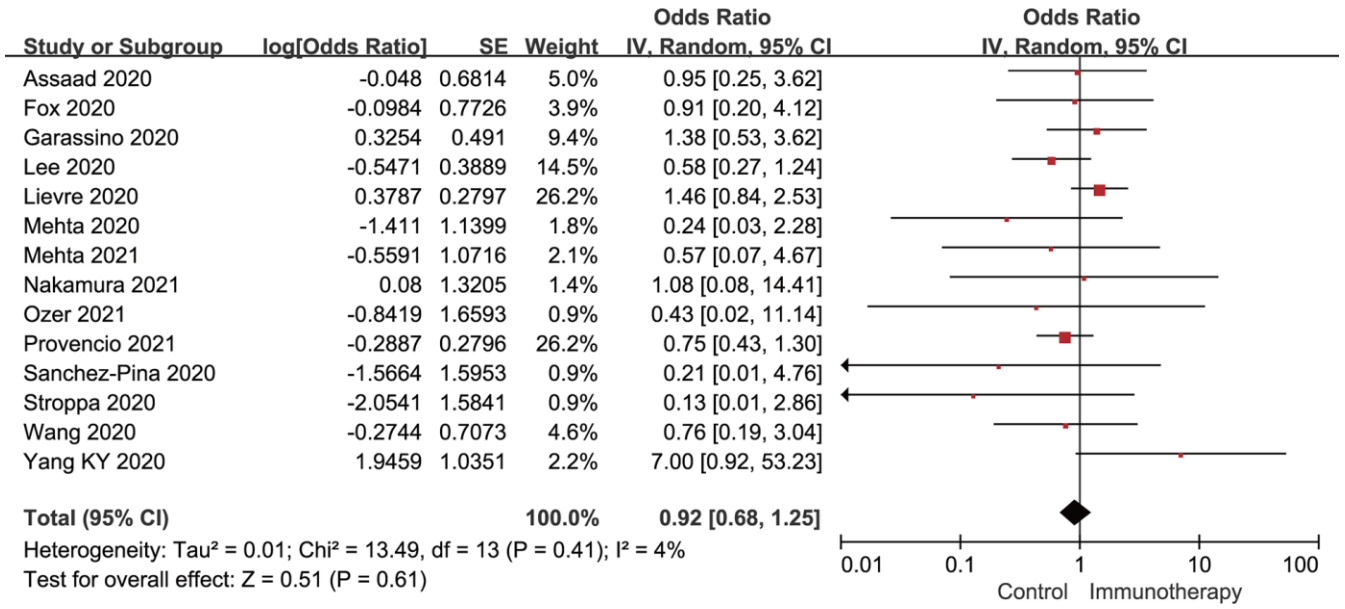

Figure 2. Forest plot of the univariate analysis for the association between active immunotherapy and mortality. CI, confidence interval; IV, inverse variance; SE, standard error.

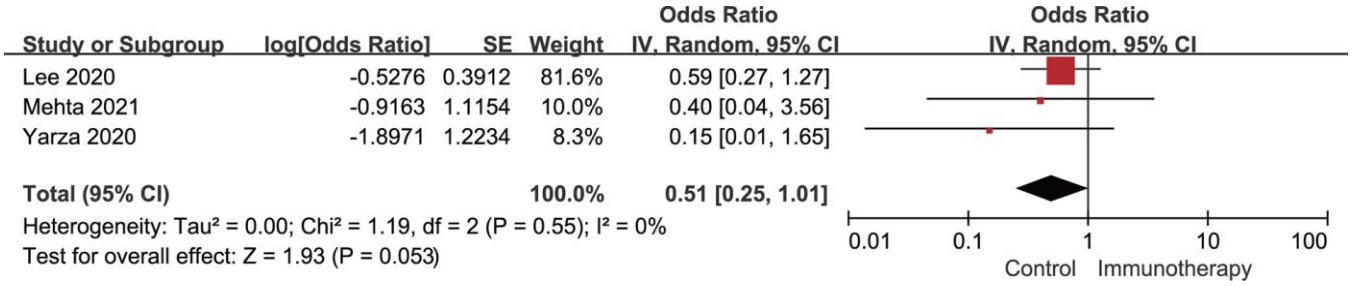

Figure 3. Forest plot of the multivariate analysis for the association between active immunotherapy and mortality. Adjusted variables for the study by Lee 2020 [22]: age, sex, and presence of comorbidities; adjusted variables for the study by Mehta 2021 [24]: age and presence of comorbidities; and adjusted variables for the study by Yarza 2020 [35]: age, sex, Eastern Cooperative Oncology Group score (ECOG), presence of metastasis, previous venous thromboembolic event (VTE), and presence of chronic obstructive pulmonary disease (COPD). CI, confidence interval; IV, inverse variance; SE, standard error.

Table 2. Meta-analyses and subgroup analyses for mortality.

Patients receiving immunotherapy vs. control patients	N of studies	Pooled OR (95%CI) [a]	I^2 (%) [b]	*P*	*P* for interaction
Overall	14	0.92 (0.68, 1.25)	4%	0.61	
Study type					
Prospective	3	0.67 (0.43, 1.04)	0%	0.07	0.054
Retrospective	11	1.19 (0.81, 1.73)	0%	0.38	
Number of patients					
<100	6	0.64 (0.28, 1.49)	0%	0.30	0.39
>100	8	0.97 (0.63, 1.49)	34%	0.90	
Cancer type					
Hematological malignancies	3	0.72 (0.27, 1.91)	0%	0.51	0.48
Solid tumor	3	1.10 (0.69, 1.76)	36%	0.68	
Non-specific cancer	8	0.72 (0.39, 1.33)	8%	0.29	
Immunotherapy interval before the COVID-19 diagnosis (days)					
>20	8	0.98 (0.56, 1.69)	25%	0.94	0.44
<20	2	1.23 (0.54, 2.76)	0%	0.62	
Number of patients receiving active immunotherapy					
<10	7	0.75 (0.27, 2.08)	22%	0.58	0.66
>10	7	0.95 (0.70, 1.28)	0%	0.73	

[a]Calculated by using the random-effect model.
[b]I^2 means the inconsistency across studies.

DISCUSSION

The present study indicated that active immunotherapy was not associated with increased mortality or rate of severe/critical disease in cancer patients who had concurrent COVID-19 infection. Some discrepancies were found between the results yielded by the present study and those yielded by the previous studies, and those studies must be updated. In the results published by Liu et al. [13], immunotherapy was found to have a tendency of increasing the risk of mortality (RR: 1.20; 95% CI: 0.68-2.13) and severe/critical disease (RR: 1.24; 95% CI: 0.94-1.63). Moreover, Liu et al. [13] indicated that immunotherapy had higher risk compared with other anticancer treatments. The variation might partially derive from the inclusion of newly published studies in the present meta-analysis. The research of Liu et al. [13] was conducted in the comparatively earlier period of the pandemic, and the number of published studies regarding COVID-19 and cancer was limited. Furthermore, the results reported by Liu et al. [13] were all unadjusted; however, the accuracy and reliability of

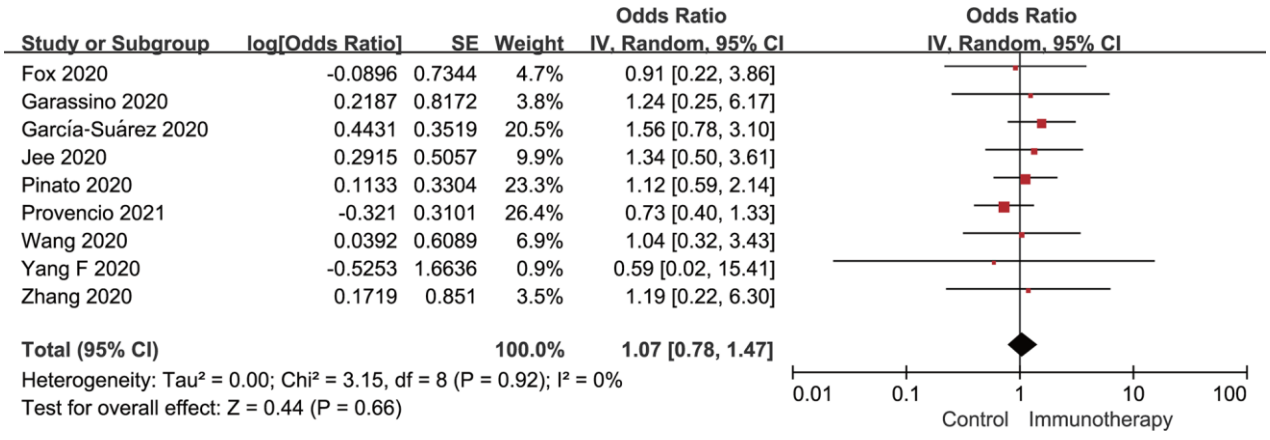

Figure 4. Forest plot of the univariate analysis for the association between active immunotherapy and severe/critical disease rate. CI, confidence interval; IV, inverse variance; SE, standard error.

these unadjusted results might be affected by a series of confounding variables (e.g., age, sex, presence of comorbidities, smoking status, presence of metastasis), particularly in retrospective studies. In the study of Yekeduz et al. [12], immunotherapy was detected to have a potential risk of increasing mortality (OR: 1.12; 95% CI: 0.60-2.08) and the rate of severe/critical disease (RR: 1.60; 95% CI: 0.72-3.52). The discrepancy might be due to the inappropriate inclusion of the study published by Dai et al. [38], in which the comparison group was patients without cancer, resulting in significantly higher heterogeneity (I^2>50%) [12].

Granted, some inconsistencies were observed, and there were still some studies supporting the present results. A previous study involving 522 patients with concurrent COVID-19 demonstrated that the number of T cells were drastically diminished in COVID-19 patients. Moreover, T cell exhaustion was also observed, concomitant with the higher expression of PD-1 and increased serum IL-6 and IL-10 in patients who were infected by COVID-19 [39]. In light of this immune response, immunotherapy administration might be conducive to patients with COVID-19 because it activates exhausted T cells by blocking PD-1/PD-L1 or CTLA-4 [40]. Furthermore, Yekeduz et al. [12] indicated that the use of immunotherapy, especially ICIs, was safe in cancer patients during the pandemic, which was in line with our results.

The present study has strengths, including comprehensive inclusion and a multivariate analysis. The figure for studies regarding immunotherapy included in this meta-analysis surpassed those in prior studies. In addition, the low publication bias and between-study heterogeneity contributed to more reliable and conservative results as well as higher quality of evidence. Moreover, in the special period of the COVID-19 outbreak, one of the major concerns is the safety of using immunotherapy to treat cancer patients, by virtue of the immune-related adverse events, which can probably lead to worsening prognosis of cancer patients who had concurrent COVID-19 infection [3]. However, the results derived from the present meta-analysis indicated that administering immunotherapy in cancer patients during the pandemic of COVID-19 was not associated with risk of death and severe/critical COVID-19. This provides more evidence for oncologists when managing cancer patients in the special era.

Some limitations should be addressed in the present research. First, the control groups were observed to be inconsistent in the included studies. Fifteen studies included cancer patients not receiving any active anticancer treatment in control groups, while the remaining five studies included cancer patients without active immunotherapy. Considering this, we conducted sensitivity analyses, of which the results showed that the exclusion of these five studies did not significantly alter the pooled results. Second, the definitions of severe/critical diseases were not totally consistent among included studies. This inconsistency might introduce the risk of bias to the present results. Third, due to the lack of adjusted results, we did not conduct a multivariate analysis of the severe/critical disease rate. The results of the univariate analysis may not mirror the real effects of immunotherapy on cancer patients as many confounders can affect the prognosis of cancer and COVID-19. Fourth, a majority of the included studies were retrospective. Fifth, some studies did not include sufficient patients on immunotherapy, which might introduce bias to the results. Although we did not find significant statistical heterogeneity or publication bias, the results yielded by the present meta-analysis should be discreetly interpreted in clinical practice, in combination with the assessment of specific patient conditions.

CONCLUSIONS

Accepting immunotherapy within 30 days before the diagnosis of COVID-19 was not significantly associated with a higher risk of mortality or severe/critical disease of infected cancer patients. Due to the limitations of the present study, the conclusions should be interpreted with discretion, and further prospectively designed studies with large sample sizes are required to evaluate the present results.

AUTHOR CONTRIBUTIONS

Chang Cao, Xinyan Gan, and Xiaolin Hu contributed equally to this article. Chang Cao, Xinyan Gan and Xiaolin Hu completed full-text screening, data extraction, and quality assessment. Xiaolin Hu and Yonglin Su designed search strategy and searched studies in electronic databases. Yu Zhang and Xingchen Peng were responsible for the study concept and design. Authors were responsible for statistical analysis.

All authors confirm that data are available on reasonable request. All data relevant to the study are included in the article or uploaded as online Supplementary Information.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Editorial note

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REFERENCES

1. WHO. WHO coronavirus disease (COVID-19) dashboard. 2021. https://covid19.who.int/

2. Kuderer NM, Choueiri TK, Shah DP, Shyr Y, Rubinstein SM, Rivera DR, Shete S, Hsu CY, Desai A, de Lima Lopes G Jr, Grivas P, Painter CA, Peters S, et al, and COVID-19 and Cancer Consortium. Clinical impact of COVID-19 on patients with cancer (CCC19): a cohort study. Lancet. 2020; 395:1907–18. https://doi.org/10.1016/S0140-6736(20)31187-9 PMID:32473681

3. Rossi E, Schinzari G, Tortora G. Pneumonitis from immune checkpoint inhibitors and COVID-19: current concern in cancer treatment. J Immunother Cancer. 2020; 8:e000952. https://doi.org/10.1136/jitc-2020-000952 PMID:32699182

4. Curigliano G, Banerjee S, Cervantes A, Garassino MC, Garrido P, Girard N, Haanen J, Jordan K, Lordick F, Machiels JP, Michielin O, Peters S, Tabernero J, et al, and Panel members. Managing cancer patients during the COVID-19 pandemic: an ESMO multidisciplinary expert consensus. Ann Oncol. 2020; 31:1320–35. https://doi.org/10.1016/j.annonc.2020.07.010 PMID:32745693

5. Desai A, Sachdeva S, Parekh T, Desai R. COVID-19 and Cancer: Lessons From a Pooled Meta-Analysis. JCO Glob Oncol. 2020; 6:557–9. https://doi.org/10.1200/GO.20.00097 PMID:32250659

6. Kim ES, Bruinooge SS, Roberts S, Ison G, Lin NU, Gore L, Uldrick TS, Lichtman SM, Roach N, Beaver JA, Sridhara R, Hesketh PJ, Denicoff AM, et al. Broadening Eligibility Criteria to Make Clinical Trials More Representative: American Society of Clinical Oncology and Friends of Cancer Research Joint Research Statement. J Clin Oncol. 2017; 35:3737–44. https://doi.org/10.1200/JCO.2017.73.7916 PMID:28968170

7. Ostios-Garcia L, Faig J, Leonardi GC, Adeni AE, Subegdjo SJ, Lydon CA, Rangachari D, Huberman MS, Sehgal K, Shea M, VanderLaan PA, Cheng MP, Marty FM, et al. Safety and Efficacy of PD-1 Inhibitors Among HIV-Positive Patients With Non-Small Cell Lung Cancer. J Thorac Oncol. 2018; 13:1037–42. https://doi.org/10.1016/j.jtho.2018.03.031 PMID:29631035

8. Ferris RL, Blumenschein G Jr, Fayette J, Guigay J, Colevas AD, Licitra L, Harrington KJ, Kasper S, Vokes EE, Even C, Worden F, Saba NF, Docampo LC, et al. Nivolumab vs investigator's choice in recurrent or metastatic squamous cell carcinoma of the head and neck: 2-year long-term survival update of CheckMate 141 with analyses by tumor PD-L1 expression. Oral Oncol. 2018; 81:45–51. https://doi.org/10.1016/j.oraloncology.2018.04.008 PMID:29884413

9. Cao C, Wei Q, Tang X, Jia Y, Sun X, Li W, Hu Q, Chen X. PD-1 and PD-L1 in locoregionally advanced nasopharyngeal carcinoma: Substudy of a randomized phase III trial. Head Neck. 2019; 41:1427–33. https://doi.org/10.1002/hed.25601 PMID:30582240

10. Kusumoto S, Arcaini L, Hong X, Jin J, Kim WS, Kwong YL, Peters MG, Tanaka Y, Zelenetz AD, Kuriki H, Fingerle-Rowson G, Nielsen T, Ueda E, et al. Risk of HBV reactivation in patients with B-cell lymphomas receiving obinutuzumab or rituximab immunochemotherapy. Blood. 2019; 133:137–46. https://doi.org/10.1182/blood-2018-04-848044 PMID:30341058

11. Tapia Rico G, Chan MM, Loo KF. The safety and efficacy of immune checkpoint inhibitors in patients with advanced cancers and pre-existing chronic viral infections (Hepatitis B/C, HIV): A review of the available evidence. Cancer Treat Rev. 2020; 86:102011. https://doi.org/10.1016/j.ctrv.2020.102011 PMID:32213376

12. Yekedüz E, Utkan G, Ürün Y. A systematic review and meta-analysis: the effect of active cancer treatment on severity of COVID-19. Eur J Cancer. 2020; 141:92–104. https://doi.org/10.1016/j.ejca.2020.09.028 PMID:33130550

13. Liu H, Yang D, Chen X, Sun Z, Zou Y, Chen C, Sun S. The effect of anticancer treatment on cancer patients with COVID-19: A systematic review and meta-analysis. Cancer Med. 2021; 10:1043–56. https://doi.org/10.1002/cam4.3692 PMID:33381923

14. WHO. Clinical management of COVID-19 patients: living guidance. 2021; https://www.who.int/publications/i/item/WHO-2019-nCoV-clinical-2021-1

15. ASCO. ASCO special report: a guide to cancer care delivery during the covid-19 pandemic. 2021. https://www.asco.org/sites/new-www.asco.org/files/content-files/2020-ASCO-Guide-Cancer-COVID19.pdf

16. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, Shamseer L, Tetzlaff JM, Akl EA, Brennan SE, Chou R, Glanville J, Grimshaw JM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 2021; 372:n71. https://doi.org/10.1136/bmj.n71 PMID:33782057

17. Stang A. Critical evaluation of the Newcastle-Ottawa scale for the assessment of the quality of nonrandomized studies in meta-analyses. Eur J Epidemiol. 2010; 25:603–5. https://doi.org/10.1007/s10654-010-9491-z PMID:20652370

18. Assaad S, Avrillon V, Fournier ML, Mastroianni B, Russias B, Swalduz A, Cassier P, Eberst L, Steineur MP, Kazes M, Perol M, Michallet AS, Rey P, et al. High mortality rate in cancer patients with symptoms of COVID-19 with or without detectable SARS-COV-2 on RT-PCR. Eur J Cancer. 2020; 135:251–9. https://doi.org/10.1016/j.ejca.2020.05.028 PMID:32540204

19. Fox TA, Troy-Barnes E, Kirkwood AA, Chan WY, Day JW, Chavda SJ, Kumar EA, David K, Tomkins O, Sanchez E, Scully M, Khwaja A, Lambert J, et al. Clinical outcomes and risk factors for severe COVID-19 in patients with haematological disorders receiving chemo- or immunotherapy. Br J Haematol. 2020; 191:194–206. https://doi.org/10.1111/bjh.17027 PMID:32678948

20. Garassino MC, Whisenant JG, Huang LC, Trama A, Torri V, Agustoni F, Baena J, Banna G, Berardi R, Bettini AC, Bria E, Brighenti M, Cadranel J, et al, and TERAVOLT investigators. COVID-19 in patients with thoracic malignancies (TERAVOLT): first results of an international, registry-based, cohort study. Lancet Oncol. 2020; 21:914–22. https://doi.org/10.1016/S1470-2045(20)30314-4 PMID:32539942

21. Jee J, Foote MB, Lumish M, Stonestrom AJ, Wills B, Narendra V, Avutu V, Murciano-Goroff YR, Chan JE, Derkach A, Philip J, Belenkaya R, Kerpelev M, et al. Chemotherapy and COVID-19 Outcomes in Patients With Cancer. J Clin Oncol. 2020; 38:3538–46. https://doi.org/10.1200/JCO.20.01307 PMID:32795225

22. Lee LY, Cazier JB, Angelis V, Arnold R, Bisht V, Campton NA, Chackathayil J, Cheng VW, Curley HM, Fittall MW, Freeman-Mills L, Gennatas S, Goel A, et al, and UK Coronavirus Monitoring Project Team. COVID-19 mortality in patients with cancer on chemotherapy or other anticancer treatments: a prospective cohort study. Lancet. 2020; 395:1919–26. https://doi.org/10.1016/S0140-6736(20)31173-9 PMID:32473682

23. Lièvre A, Turpin A, Ray-Coquard I, Le Malicot K, Thariat J, Ahle G, Neuzillet C, Paoletti X, Bouché O, Aldabbagh K, Michel P, Debieuvre D, Canellas A, et al, and GCO-002 CACOVID-19 collaborators/investigators. Risk factors for Coronavirus Disease 2019 (COVID-19) severity and mortality among solid cancer patients and impact of the disease on anticancer treatment: A French nationwide cohort study (GCO-002 CACOVID-19). Eur J Cancer. 2020; 141:62–81. https://doi.org/10.1016/j.ejca.2020.09.035 PMID:33129039

24. Mehta A, Vasudevan S, Parkash A, Sharma A, Vashist T, Krishna V. COVID-19 mortality in cancer patients: a report from a tertiary cancer centre in India. PeerJ. 2021; 9:e10599. https://doi.org/10.7717/peerj.10599 PMID:33552716

25. Mehta V, Goel S, Kabarriti R, Cole D, Goldfinger M, Acuna-Villaorduna A, Pradhan K, Thota R, Reissman S, Sparano JA, Gartrell BA, Smith RV, Ohri N, et al. Case Fatality Rate of Cancer Patients with COVID-19 in a New York Hospital System. Cancer Discov. 2020; 10:935–41. https://doi.org/10.1158/2159-8290.CD-20-0516 PMID:32357994

26. Nakamura S, Kanemasa Y, Atsuta Y, Fujiwara S, Tanaka M, Fukushima K, Kobayashi T, Shimoyama T, Omuro Y, Sekiya N, Imamura A. Characteristics and outcomes of coronavirus disease 2019 (COVID-19) patients with cancer: a single-center retrospective observational study in Tokyo, Japan. Int J Clin Oncol. 2021; 26:485–93. https://doi.org/10.1007/s10147-020-01837-0 PMID:33225396

27. Ozer M, Goksu SY, Mahdi M, Gandhi N. Characteristics and outcomes of cancer patients with covid-19 at a safety-net hospital. Cancer Treat Res Commun. 2021; 28:100418. https://doi.org/10.1016/j.ctarc.2021.100418 PMID:34118790

28. Pinato DJ, Zambelli A, Aguilar-Company J, Bower M, Sng C, Salazar R, Bertuzzi A, Brunet J, Mesia R, Segui E, Biello F, Generali D, Grisanti S, et al. Clinical portrait of the SARS-CoV-2 epidemic in European cancer patients. Cancer Discov. 2020; 10:1465–74. https://doi.org/10.1158/2159-8290.CD-20-0773 PMID:32737082

29. Provencio M, Mazarico Gallego JM, Calles A, Antoñanzas M, Pangua C, Mielgo Rubio X, Nadal E, Castro RL, López-Martín A, Del Barco E, Dómine M, Franco F, Diz P, et al. Lung cancer patients with COVID-19 in Spain: GRAVID study. Lung Cancer. 2021; 157:109–15. https://doi.org/10.1016/j.lungcan.2021.05.014 PMID:34016490

30. Sanchez-Pina JM, Rodríguez Rodriguez M, Castro Quismondo N, Gil Manso R, Colmenares R, Gil Alos D, Paciello ML, Zafra D, Garcia-Sanchez C, Villegas C, Cuellar C, Carreño-Tarragona G, Zamanillo I, et al. Clinical course and risk factors for mortality from COVID-19 in patients with haematological malignancies. Eur J Haematol. 2020; 105:597–607. https://doi.org/10.1111/ejh.13493 PMID:32710500

31. Stroppa EM, Toscani I, Citterio C, Anselmi E, Zaffignani E, Codeluppi M, Cavanna L. Coronavirus disease-2019 in cancer patients. A report of the first 25 cancer patients in a western country (Italy). Future Oncol. 2020; 16:1425–32. https://doi.org/10.2217/fon-2020-0369 PMID:32403946

32. Wang B, Van Oekelen O, Mouhieddine TH, Del Valle DM, Richter J, Cho HJ, Richard S, Chari A, Gnjatic S, Merad M, Jagannath S, Parekh S, Madduri D. A tertiary center experience of multiple myeloma patients with COVID-19: lessons learned and the path forward. J Hematol Oncol. 2020; 13:94. https://doi.org/10.1186/s13045-020-00934-x PMID:32664919

33. Yang F, Shi S, Zhu J, Shi J, Dai K, Chen X. Clinical characteristics and outcomes of cancer patients with COVID-19. J Med Virol. 2020; 92:2067–73. https://doi.org/10.1002/jmv.25972 PMID:32369209

34. Yang K, Sheng Y, Huang C, Jin Y, Xiong N, Jiang K, Lu H, Liu J, Yang J, Dong Y, Pan D, Shu C, Li J, et al. Clinical characteristics, outcomes, and risk factors for mortality in patients with cancer and COVID-19 in Hubei, China: a multicentre, retrospective, cohort study. Lancet Oncol. 2020; 21:904–13. https://doi.org/10.1016/S1470-2045(20)30310-7 PMID:32479787

35. Yarza R, Bover M, Paredes D, López-López F, Jara-Casas D, Castelo-Loureiro A, Baena J, Mazarico JM, Folgueira MD, Meléndez-Carmona MÁ, Reyes A, Lumbreras C, Paz-Ares L, et al. SARS-CoV-2 infection in cancer patients undergoing active treatment: analysis of clinical features and predictive factors for severe respiratory failure and death. Eur J Cancer. 2020; 135:242–50. https://doi.org/10.1016/j.ejca.2020.06.001 PMID:32586724

36. Zhang H, Wang L, Chen Y, Wu Q, Chen G, Shen X, Wang Q, Yan Y, Yu Y, Zhong Y, Wang X, Chua ML, Xie C. Outcomes of novel coronavirus disease 2019 (COVID-19) infection in 107 patients with cancer from Wuhan, China. Cancer. 2020; 126:4023–31. https://doi.org/10.1002/cncr.33042 PMID:32573776

37. García-Suárez J, de la Cruz J, Cedillo Á, Llamas P, Duarte R, Jiménez-Yuste V, Hernández-Rivas JÁ, Gil-Manso R, Kwon M, Sánchez-Godoy P, Martínez-Barranco P, Colás-Lahuerta B, Herrera P, et al, and Asociación Madrileña de Hematología y Hemoterapia (AMHH). Impact of hematologic malignancy and type of cancer therapy on COVID-19 severity and mortality: lessons from a large population-based registry study. J Hematol Oncol. 2020; 13:133. https://doi.org/10.1186/s13045-020-00970-7 PMID:33032660

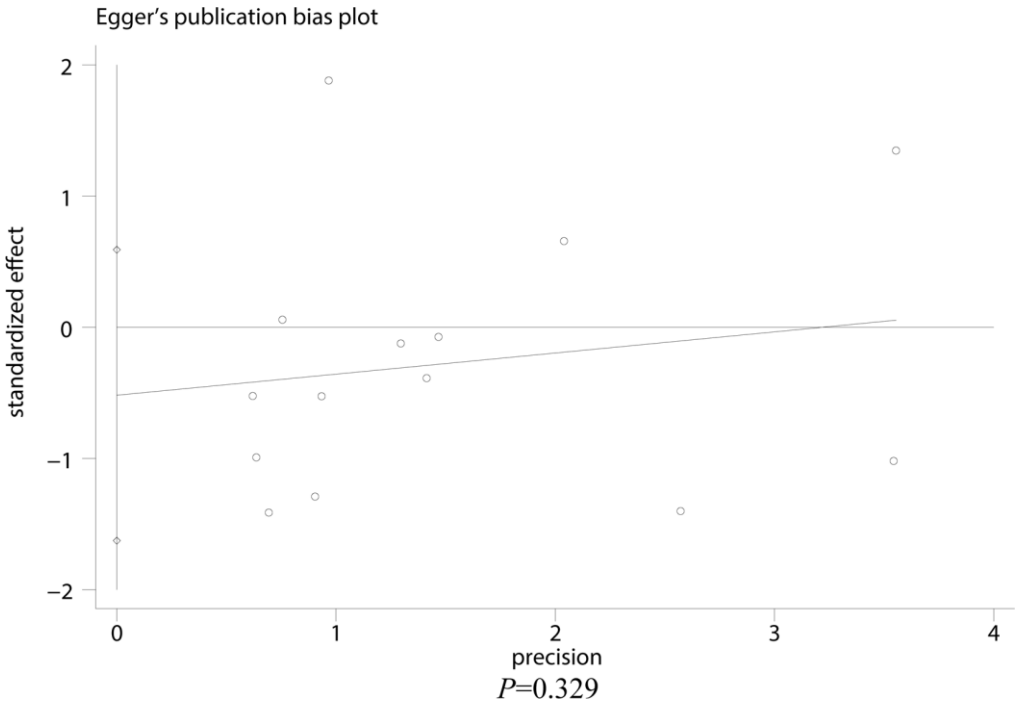
38. Dai M, Liu D, Liu M, Zhou F, Li G, Chen Z, Zhang Z, You H, Wu M, Zheng Q, Xiong Y, Xiong H, Wang C, et al. Patients with Cancer Appear More Vulnerable to SARS-CoV-2: A Multicenter Study during the COVID-19 Outbreak. Cancer Discov. 2020; 10:783–91. https://doi.org/10.1158/2159-8290.CD-20-0422 PMID:32345594

39. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, Chen L, Li M, Liu Y, Wang G, Yuan Z, Feng Z, Zhang Y, et al. Reduction and Functional Exhaustion of T Cells in Patients With Coronavirus Disease 2019 (COVID-19). Front Immunol. 2020; 11:827. https://doi.org/10.3389/fimmu.2020.00827 PMID:32425950

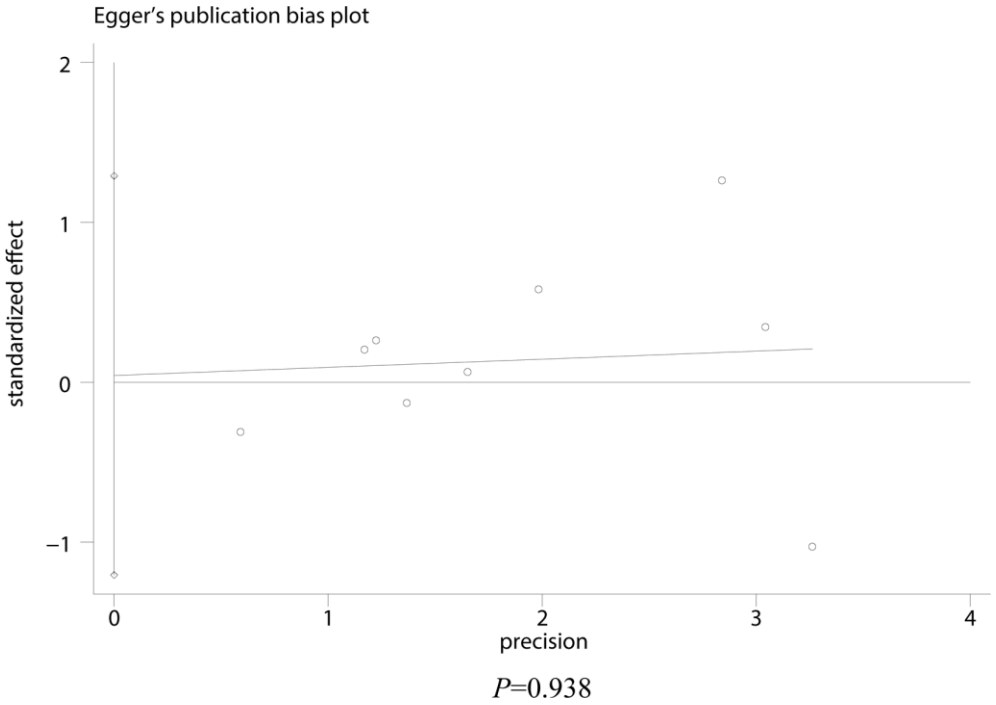
40. Hamashima R, Uchino J, Morimoto Y, Iwasaku M, Kaneko Y, Yamada T, Takayama K. Association of immune checkpoint inhibitors with respiratory infections: A review. Cancer Treat Rev. 2020; 90:102109. https://doi.org/10.1016/j.ctrv.2020.102109 PMID:33038863

SUPPLEMENTARY MATERIAL

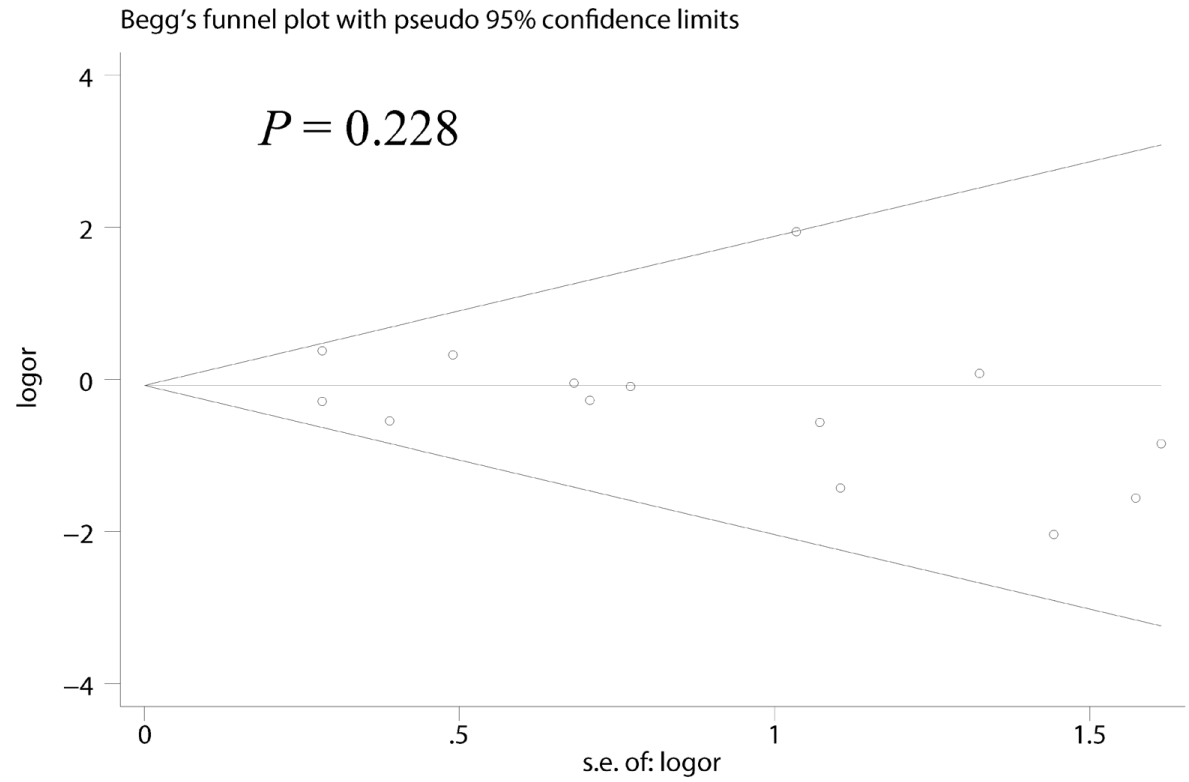
Supplementary Figures



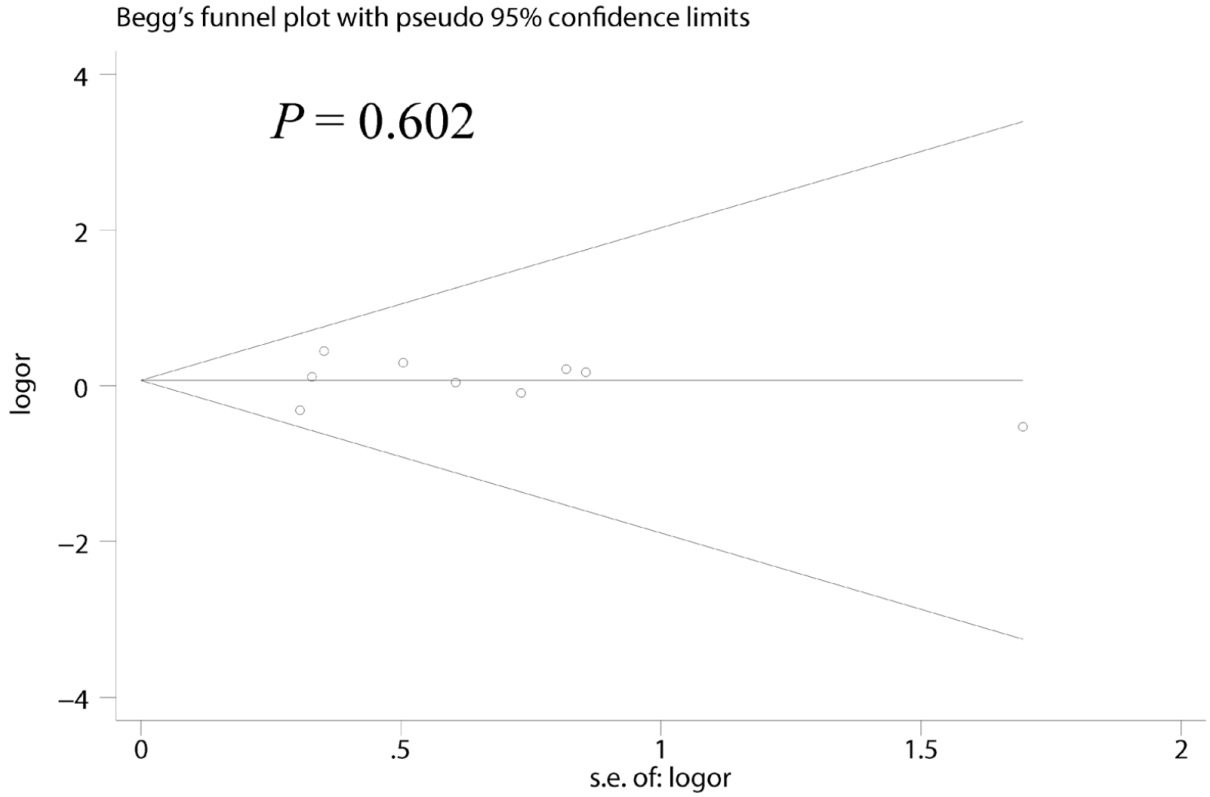
Supplementary Figure 1. Publication bias of studies regarding mortality (Egger's linear regression test).



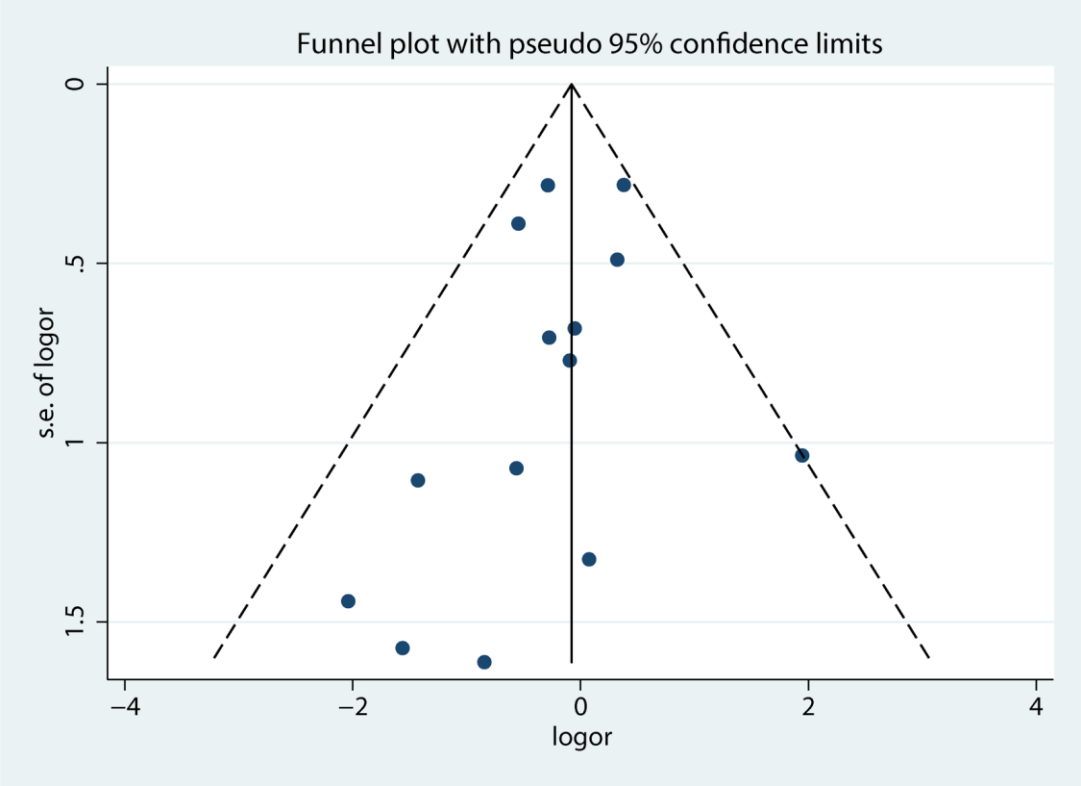
Supplementary Figure 2. Publication bias of studies regarding severe/critical disease (Egger's linear regression test).



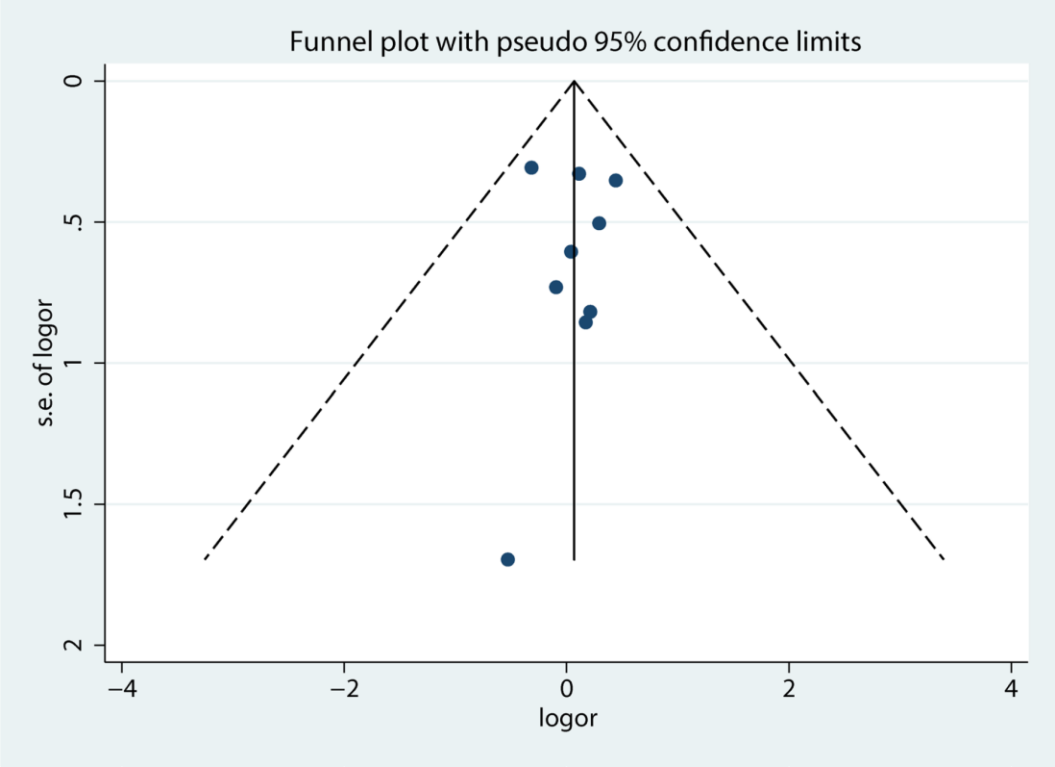
Supplementary Figure 3. Publication bias of studies regarding mortality (Begg's rank correlation test).



Supplementary Figure 4. Publication bias of studies regarding severe/critical disease (Begg's rank correlation test).



Supplementary Figure 5. Publication bias of studies regarding mortality (funnel plot).



Supplementary Figure 6. Publication bias of studies regarding severe/critical disease (funnel plot).

Supplementary Tables

Supplementary Table 1. Search strategy.

	Embase	
1	exp immunotherapy/	58400
2	(immunotherapy or immunotherapies or immunotherapeutic or immunotherapeutics or immuno* or immune* or immunothera* or ICI or ICIs or CPI or immune-checkpoint inhibitor or immune checkpoint inhibitor or (immune adj2 checkpoint) or immune-checkpoint blockade or immune checkpoint blockade or immune?checkpoint* or nivolumab or pembrolizumab or atezolizumab or avelumab or durvalumab or ipilimumab or PD-1 or PD-L1 or (PD adj3 immunotherapy) or CTLA-4 or CTLA?4).mp.	703128
3	exp cancer/	641207
4	(tumor or carcinoma or cancer or malignant or malignancy or malignan* or neoplasia or neoplasm or neoplastic or neopla* or carcinoma or carcinomatous or carcino* or adenocarcinoma or metastic or metastases or metastasis or oncology or oncological or hematology or hematolog* or haematolog* or leukemia or lymphoma or myeloma).mp.	993990
5	exp COVID-19/	145801
6	(COVID-19 or SARS-CoV-2 or Novel coronavirus or Wuhan coronavirus or 2019 coronavirus or COVID or pandemic).mp.	180887
7	(1 or 2) and (3 or 4) and (5 or 6)	3238
8	exp humans/ not animals.sh.	3415527
9	7 and 8	2895
	PubMed	
1	immunotherapy	40,439
2	immunotherapy or immunotherapies or immunotherapeutic or immunotherapeutics or ICI or ICIs or CPI or immune-checkpoint inhibitor or immune checkpoint inhibitor or immune-checkpoint blockade or immune checkpoint blockade or nivolumab or pembrolizumab or atezolizumab or avelumab or durvalumab or ipilimumab or PD-1 or PD-L1 or CTLA-4	52,369
3	cancer	414,013
4	tumor or carcinoma or cancer or malignant or malignancy or neoplasia or neoplasm or neoplastic or carcinoma or carcinomatous or adenocarcinoma or metastic or metastases or metastasis or oncology or oncological or hematology or leukemia or lymphoma or myeloma	738,253
5	COVID-19	166,753
6	COVID-19 or SARS-CoV-2 or Novel coronavirus or Wuhan coronavirus or 2019 coronavirus or COVID or pandemic	171,755
7	(1 or 2) and (3 or 4) and (5 or 6)	3785
8	Animals [Title/Abstract]	57,424
9	7 not 8	1728
	Web of science	
1	TS=(immunotherapy)	35,556
2	TS=(immunotherapy or immunotherapies or immunotherapeutic or immunotherapeutics or immuno* or immune* or immunothera* or ICI or ices or CPI or immune-checkpoint inhibitor or immune checkpoint inhibitor or immune-checkpoint blockade or immune checkpoint blockade or immune?checkpoint* or nivolumab or pembrolizumab or atezolizumab or avelumab or durvalumab or ipilimumab or PD-1 or PD-L1 or CTLA-4)	602,701
3	TS=(cancer)	447,446
4	TS=(tumor or carcinoma or cancer or malignant or malignancy or malignan* or neoplasia or neoplasm or neoplastic or neopla* or carcinoma or carcinomatous or carcino* or adenocarcinoma or metastic or metastases or metastasis or oncology or oncological or hematology or hematolog* or haematolog* or leukemia or lymphoma or myeloma)	765,618
5	TS=(COVID-19)	191,781
6	TS=(COVID-19 or SARS-CoV-2 or Novel coronavirus or Wuhan coronavirus or 2019 coronavirus or	212,159

	COVID or pandemic)	
7	(1 or 2) and (3 or 4) and (5 or 6)	4196
8	TS=(animals)	1,440,043
9	7 not 8	1258
	EBM reviews – cochrane central register of controlled trials	
1	exp immunotherapy/	671
2	(immunotherapy or immunotherapies or immunotherapeutic or immunotherapeutics or immuno* or immune* or immunothera* or ICI or ICIs or CPI or immune-checkpoint inhibitor or immune checkpoint inhibitor or (immune adj2 checkpoint) or immune-checkpoint blockade or immune checkpoint blockade or immune?checkpoint* or nivolumab or pembrolizumab or atezolizumab or avelumab or durvalumab or ipilimumab or PD-1 or PD-L1 or (PD adj3 immunotherapy) or CTLA-4 or CTLA?4).mp.	22574
3	exp neoplasms/	9971
4	(tumor or carcinoma or cancer or malignant or malignancy or malignan* or neoplasia or neoplasm or neoplastic or neopla* or carcinoma or carcinomatous or carcino* or adenocarcinoma or metastic or metastases or metastasis or oncology or oncological or hematology or hematolog* or haematolog* or leukemia or lymphoma or myeloma).mp.	42082
5	(COVID-19 or SARS-CoV-2 or Novel coronavirus or Wuhan coronavirus or 2019 coronavirus or COVID or pandemic).mp.	7342
6	(1 or 2) and (3 or 4) and 5	202
	China national knowledge infrastructure	
1	immunotherapy	26,785
2	immunotherapy or immune-checkpoint inhibitor or PD-1 or PD-L1 or CTLA-4	39,239
3	cancer	368,546
4	cancer or tumor or blood or leukemia or lymphoma	741,583
5	COVID-19	25,561
6	COVID-19 or SARS-CoV-2	36,920
7	(1 or 2) and (3 or 4) and (5 or 6)	153

Supplementary Table 2. List of articles assessed for eligibility.

ID	DOI	Include	Reason of exclusion
Assaad 2020	10.1016/j.ejca.2020.05.028	√	
Fox 2020	10.1111/bjh.17027	√	
Garassino 2020	10.1016/S1470-2045(20)30314-4	√	
García-Suárez 2020	10.1186/s13045-020-00970-7	√	
Jee 2020	10.1200/JCO.20.01307	√	
Lee 2020	10.1016/S0140-6736(20)31173-9	√	
Lievre 2020	10.1016/j.ejca.2020.09.035	√	
Mehta 2020	10.1158/2159-8290.CD-20-0516	√	
Mehta 2021	10.7717/peerj.10599	√	
Nakamura 2021	10.1007/s10147-020-01837-0	√	
Ozer 2021	10.1016/j.ctarc.2021.100418	√	
Pinato 2020	10.1158/2159-8290.CD-20-0773	√	
Provencio 2021	10.1016/j.lungcan.2021.05.014	√	
Sanchez-Pina 2020	10.1111/ejh.13493	√	
Stroppa 2020	10.2217/fon-2020-0369	√	
Wang 2020	10.1186/s13045-020-00934-x	√	
Yang F 2020	10.1002/jmv.25972	√	
Yang KY 2020	10.1016/S1470-2045(20)30310-7	√	
Yarza 2020	10.1016/j.ejca.2020.06.001	√	
Zhang 2020	10.1002/cncr.33042	√	
Dai 2020	10.1158/2159-8290.CD-20-0422		√ (included patients without cancer in the comparison group)
Fu 2021	10.1002/cncr.33657		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Lara 2020	10.1002/cncr.33084,		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Luo 2020	10.1016/j.annonc.2020.06.007		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Martin 2021	10.1002/onco.13831		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Robilotti 2020	10.1038/s41591-020-0979-0		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Russell 2020	10.3389/fonc.2020.01279		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Song 2021	10.1002/cncr.		√ (intervals between immunotherapy and diagnosis of COVID-19 > 30d)
Ali 2020	10.1016/j.hemonc.2020.12.001		√ (did not report the outcomes of patients receiving active immunotherapy)
Booth 2020	10.1111/EJH.13469		√ (did not report the outcomes of patients receiving active immunotherapy)
Caffo 2020	10.1016/j.ejca.2020.09.018		√ (did not report the outcomes of patients receiving active immunotherapy)
Cattaneo 2020	10.1002/cncr.33160		√ (did not report the outcomes of patients receiving active immunotherapy)
Di Cosimo 2021	10.3390/cancers13061324		√ (did not report the outcomes of patients receiving active immunotherapy)
Guarneri 2021	10.1016/j.ejca.2021.01.021		√ (did not report the outcomes of patients receiving active immunotherapy)
Kuderer 2020	10.1016/S0140-6736(20)31187-9		√ (did not report the outcomes of patients receiving active immunotherapy)
Li 2020	10.1038/s41375-020-0986-7		√ (did not report the outcomes of patients receiving active immunotherapy)
Liang 2021	10.1007/s11684-021-0845-6		√ (did not report the outcomes of patients receiving active immunotherapy)
Liu 2020	10.1136/jitc-2020-001314		√ (did not report the outcomes of patients receiving active immunotherapy)
Liu 2021	10.7150/jca.54205		√ (did not report the outcomes of patients receiving active immunotherapy)
Ma 2020	10.1016/j.jinf.2020.04.006		√ (did not report the outcomes of patients receiving active immunotherapy)
Martín-Moro 2020	10.1111/bjh.16801		√ (did not report the outcomes of patients receiving active immunotherapy)
Mato 2020	10.1182/blood.2020006965.		√ (did not report the outcomes of patients receiving active immunotherapy)
Morais 2021	10.1002/ijc.33532		√ (did not report the outcomes of patients receiving active immunotherapy)
Nicole 2020	10.1016/S0140-6736(20)31187-9		√ (did not report the outcomes of patients receiving active immunotherapy)
Rogado 2020	10.1007/s12094-020-02381-z		√ (did not report the outcomes of patients receiving active immunotherapy)
Scarfò 2020	10.1038/s41375-020-0959-x		√ (did not report the outcomes of patients receiving active immunotherapy)
Tian 2020	10.1016/S1470-2045(20)30309-0		√ (did not report the outcomes of patients receiving active immunotherapy)
Vuagnat 2020	10.1186/s13058-020-01293-8		√ (did not report the outcomes of patients receiving active immunotherapy)
Wei 2021	10.1016/j.breast.2021.06.006		√ (did not report the outcomes of patients receiving active immunotherapy)
Zhang 2020	10.1016/j.annonc.2020.03.296		√ (did not report the outcomes of patients receiving active immunotherapy)

Argenziano 2020	10.1136/bmj.m1996	√ (did not report the outcomes of patients receiving active immunotherapy)
Feng 2020	10.1164/rccm.202002-0445OC	√ (did not report the outcomes of patients receiving active immunotherapy)
Gill 2021	10.1371/journal.pone.0248498	√ (did not report the outcomes of patients receiving active immunotherapy)
Huang 2020	10.1016/S0140-6736(20)30183-5	√ (did not report the outcomes of patients receiving active immunotherapy)

Supplementary Table 3. Quality assessment of included studies.

ID	Selection	Comparability	Exposure/Outcome	Score
Assaad 2020	**	*	***	6
Fox 2020	**	*	***	6
Garassino 2020	**	**	***	7
García-Suárez 2020	***	*	***	7
Jee 2020	**	*	***	6
Lee 2020#	***	*	***	7
Lievre 2020	***	*	***	7
Mehta 2020	**	*	***	6
Mehta 2021	**	**	***	7
Nakamura 2021	**	**	***	7
Ozer 2021	***	*	***	7
Pinato 2020	**	*	***	6
Provencio 2021[#]	***	**	***	8
Sanchez-Pina 2020	**	*	***	6
Stroppa 2020	**	*	***	6
Wang 2020	**	*	***	6
Yang F 2020	**	*	***	6
Yang KY 2020	***	**	***	8
Yarza 2020#	****	*	***	8
Zhang 2020	***	*	***	7

[#] Quality assessment performed by using Newcastle-Ottawa scale (NOS) for cohort studies. Remainder assessed by using the NOS for case-control studies.

Supplementary Table 4. Meta-regression of studies regarding mortality.[a]

Covariate	Estimate	Standard error	t-value	p-value	Estimate LCI[b]	Estimate UCI[c]
Study type	-0.6473	0.4449	-1.4547	0.1838	-1.6733	0.3788
Number of patients	0.0002	0.0004	0.551	0.5967	-0.0007	0.0012
Cancer type	-0.0759	0.427	-0.1777	0.8634	-1.0605	0.9088
Immunotherapy interval before COVID-19 diagnosis	-0.0183	0.0273	-0.6698	0.5218	-0.0811	0.0446
Number of patients receiving active immunotherapy	0.0061	0.0117	0.5262	0.613	-0.0208	0.033

[a] The robustness of meta-regression is validated by permutation test.
[b] LCI means lower bound of 95% confidence interval.
[c] UCI means upper bound of 95% confidence interval.

Supplementary Table 5. Meta-regression of studies regarding severe/critical disease.[a]

Covariate	Estimate	Standard error	*t*-value	*p*-value	Estimate LCI[b]	Estimate UCI[c]
Study type	0.2028	0.2288	0.8864	0.4407	-0.5253	0.9309
Number of patients	0.0006	0.0003	1.9959	0.1399	-0.0004	0.0015
Cancer type	-0.0385	0.1411	-0.2727	0.8028	-0.4876	0.4106
Immunotherapy interval before COVID-19 diagnosis	0.0025	0.0117	0.2107	0.8466	-0.0347	0.0396
Number of patients receiving active immunotherapy	-0.0103	0.0055	-1.8596	0.1599	-0.0278	0.0073

[a]The robustness of meta-regression is validated by permutation test.
[b]LCI means lower bound of 95% confidence interval.
[c]UCI means upper bound of 95% confidence interval.

Research Paper

Association between social isolation and reduced mental well-being in Swedish older adults during the first wave of the COVID-19 pandemic: the role of cardiometabolic diseases

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ABSTRACT

Social isolation has been recommended as a strategy for reducing COVID-19 risk, but it may have unintended consequences for mental well-being. We explored the relationship between social isolation and symptoms of depression and anxiety in older adults during the first wave of the COVID-19 pandemic and assessed the role of cardiometabolic diseases (CMDs) in this association. Between May and September 2020, 1,190 older adults from the Swedish National Study on Aging and Care in Kungsholmen were surveyed about their behaviors and health consequences during the first wave of the COVID-19 pandemic. In total, 913 (76.7%) participants reported socially isolating at home to avoid infection during this period. Social isolation was associated with a greater likelihood of reduced mental well-being (i.e., feelings of depression or anxiety) (OR: 1.74, 95% CI: 1.15-2.65). In joint exposure analysis, there was a significant likelihood of reduced mental well-being only among people who were socially isolating and had CMDs (OR: 2.13, 95% CI: 1.22-3.71) (reference: not isolating, CMD-free). In conclusion, social isolation as a COVID-19 prevention strategy was related to reduced mental well-being in an urban sample of Swedish older adults, especially among individuals with CMDs.

INTRODUCTION

In early 2020, a highly infectious novel coronavirus (SARS-CoV2) began to spread across the world, leading the World Health Organization to declare COVID-19, the respiratory disease caused by the virus, an international public health emergency on January 30 and a global pandemic on March 11 [1]. Advanced age is a key risk factor for COVID-19 mortality [2]. Other factors associated with COVID-19 fatality include male sex and the presence of cardiometabolic comorbidities such as type 2 diabetes (T2D) and cardiovascular disease (CVD) [3, 4].

In an effort to curb the virus' spread and reduce COVID-19 mortality, many countries implemented strict measures, including lockdowns and stay-at-home orders. In contrast, Sweden's pandemic response emphasized voluntary adherence to recommendations from the Public Health Agency, including avoiding contact with others if one showed signs of COVID-19 symptoms, maintaining hand hygiene, and practicing

social distancing [5]. Additionally, older adults in Sweden were further advised to stay at home and avoid crowded venues such as social gatherings and public transportation [5].

Though it is undoubtably effective for reducing the spread of infection, social isolation – that is, staying at home and minimizing in-person contact with others – may have unintended negative consequences. Before the COVID-19 pandemic, social isolation has been identified as a risk factor for poorer mental health [6, 7]. Consistent with this, recent studies have linked social isolation as a COVID-19 prevention measure with reduced mental health, both in the general adult population [8–10] and among older and more frail adults in particular [11–13].

Despite increased interest in the impact of pandemic-related social isolation, no studies to our knowledge have explored this issue in relation to the growing population of older adults with cardiometabolic disease. On one hand, people with cardiometabolic diseases (CMDs) stand to benefit most from social isolation from a COVID-19 prevention perspective, given the higher COVID-19 fatality rates associated with T2D and CVD [3]. On the other hand, other possible negative health consequences (e.g., reduced physical activity and cognitive decline) that accompany social isolation could be particularly damaging in this already-vulnerable population.

Pandemics are predicted to become more intense and more frequent with continued globalization and expansion. To this end, a recent investigation estimated that the probability of a COVID-19-scale pandemic is as high as 2% in any given year [14]. For the remainder of the current pandemic as well as for inevitable future ones, it is important to assess what is lost and gained by social isolation, particularly for vulnerable populations.

In this study, using data from the Swedish National Study on Aging and Care in Kungsholmen (SNAC-K), we aimed to assess the association of social isolation with depression and anxiety during the COVID-19 pandemic, and to examine the role of CMDs in this association. We hypothesized that social isolation would have an adverse impact on mental health and that these associations may be modified by the presence of CMDs.

RESULTS

Characteristics of the study population

Of 1,190 study participants (64.0% female, mean age 78.6 ± 8.2 years), 913 (76.7%) had been socially isolating during the first wave of the COVID-19 pandemic. Compared to those who did not isolate, socially isolating participants were more likely to be older, have a lower education level, and have CMDs, but were less likely to smoke or drink heavily (Table 1).

Associations between social isolation status and mental well-being

Participants who had been socially isolating showed a higher likelihood of reduced mental well-being (multi-adjusted OR [95% CI]: 1.74 [1.15 – 2.65]) compared to those who were not isolating, including a higher likelihood of both depressive (2.08 [1.10 – 3.94]) and anxiety (1.82 [1.16 – 2.84]) symptoms (Table 2).

Joint effect of social isolation status and CMDs on mental well-being

The presence of CMDs was not significantly associated with reduced mental well-being (OR 1.30, 95% CI 0.88 – 1.93) compared to those without CMDs. However, in joint exposure analysis, compared to CMD-free participants who were not socially isolating, participants who were socially isolating and had CMDs had over twice the likelihood of reduced mental well-being (OR 2,13, 95% CI 1.22 – 3.71). Similarly, there was a significantly increased likelihood (OR [95% CI]) of depressive (3.34 [1.50 – 7.45]) and anxiety (2.05 [1.13 – 3.70]) symptoms only among participants who both had CMDs and were socially isolating (Figure 1). There was a significant additive (p=0.02) but not multiplicative (p=0.208) interaction between social isolation status and CMDs on the likelihood of reduced mental well-being.

Gender-specific effects of social isolation and CMDs on mental well-being

After stratifying by gender, the association between self-isolation and reduced mental well-being (OR, 95% CI) was statistically significant among women (1.80, 1.09-2.96). Moreover, in joint exposure analysis, the OR (95% CI) of reduced mental well-being was 2.26 (1.16-4.42) among women who were isolating and had CMDs compared to those who were CMD-free and not isolating. Among men, these associations had a similar effect size, but were not statistically significant (Table 3). There were no significant interactions, additive or multiplicative, between social isolation status and CMDs for men and women separately.

DISCUSSION

In this population-based cohort study of older adults in Stockholm during the first wave of the COVID-19 pandemic, we found that: 1) social isolation was

Table 1. Baseline characteristics of the study population (n = 1190).

Variables	Total	Social isolation status		
		Not isolating (n=277)	Isolating (n=913)	P value
Age (years)	78.6±8.2	74.4±7.2	79.9±8.1	<0.001
<80 years	623 (52.4)	205 (74.0)	418 (45.8)	<0.001
≥80 years	567 (47.7)	72 (26.0)	595 (54.2)	
Gender				
Men	429 (36.0)	109 (39.4)	320 (35.1)	0.192
Women	761 (64.0)	168 (60.7)	593 (65.0)	
Education				
Elementary	41 (3.5)	4 (1.4)	37 (4.1)	0.031
High school	460 (38.7)	98 (35.4)	362 (39.7)	
University	698 (57.9)	175 (63.2)	514 (56.3)	
Living alone	589 (49.6)	134 (48.4)	455 (50.0)	0.647
Smoking				
Never	826 (90.7)	190 (82.6)	636 (93.4)	<0.001
Former smoker	22 (2.4)	13 (5.7)	9 (1.3)	
Current smoker	63 6.9)	27 (11.7)	36 (5.3)	
Alcohol consumption				
No or occasional	192 (21.9)	27 (12.2)	165 (25.3)	<0.001
Light to moderate	507 (57.9)	140 (63.1)	367 (56.2)	
Heavy	176 (20.1)	55 (24.8)	121 (18.5)	
Pre-pandemic depressive symptoms score	2.0±2.7	2.0±2.6	2.5±3.2	0.0639
Cardiometabolic diseases	277 (23.3)	43 (15.5)	234 (25.6)	<0.001
Reduced mental health during the first wave of the COVID-19 pandemic	290 (24.4)	55 (19.9)	235 (25.7)	0.046
Depression	110 (9.2)	19 (6.9)	91 (9.10)	0.118
Anxiety	245 (20.6)	44 (15.9)	201 (22.0)	0.027

Data are presented as means ± standard deviations or number (proportion %).
Missing variables: 2 were missing data on living conditions, 279 on smoking status, 315 on alcohol consumption, and 276 on depressive symptoms score.

Table 2. Relationship between social isolation and mental health among older adults during the first wave of the COVID-19 pandemic.

	n	Reduced mental well-being		Components of reduced mental well-being			
				Depressive symptoms		Anxiety symptoms	
		OR (95% CI)*	*P*-value	OR (95% CI)*	*P*-value	OR (95% CI)*	*P*-value
Social isolation							
No	277	Reference		Reference		Reference	
Yes	913	1.74 (1.15 – 2.65)	0.009	2.08 (1.10 – 3.94)	0.024	1.82 (1.16 – 2.84)	0.009

*Logistic regression model adjusted for baseline age, gender, education, living status, smoking status, alcohol consumption, and pre-pandemic depressive symptoms.

associated with higher levels of depression and anxiety and 2) reduced mental well-being was greatest among participants who were socially isolating and had CMDs.

Social isolation is a widely recognized risk factor for depression and anxiety and its effect on mental health may be particularly pronounced among older adults [6, 7]. The consequences of social isolation are particularly important to consider in the context of the COVID-19 pandemic, which forced people around the world into isolation at home on an unprecedented scale. While the full extent of the pandemic's impact on mental health is still coming into view, studies conducted in the midst of the COVID-19 outbreak

indicate that engaging in social isolation to avoid infection comes with significant collateral damage. The COVID-19 Mental Disorders Collaboration reported that reduced human mobility during the pandemic (calculated using mobile phone user data and information on physical distancing mandates) was associated with an increase in the prevalence of major depressive disorder and anxiety disorders globally [10]. A study including nearly 10,000 participants across 78 countries uncovered high levels of reduced mental health during the pandemic, but this was buffered by leaving home more often [9]. Another study reported a correlation between depressive symptoms and more days stayed at home during the pandemic [8]. Finally, a comprehensive review on the mental and physical effects of the pandemic on older people described consistent negative impacts of social distancing and social isolation on mental well-being – in particular, depression, anxiety, and reduced sleep quality [12].

Consistent with these reports, we found that social isolation was related to reduced mental well-being in the form of feelings of both depression and anxiety in an urban population of Swedish older adults. It is notable that these adverse effects of social isolation were apparent even in the context of Sweden's comparatively relaxed COVID-19 prevention measures, where people who isolated themselves at home did so by individual choice and not because of an external mandate. Additional studies are needed to better understand the long-term impacts of COVID-19-related social isolation beyond the pandemic's first wave.

Our study takes the further step of examining the impact of cardiometabolic disease on the association between social isolation and mental well-being. Older adults with CMDs like T2D and CVD have far greater risk of COVID-19 fatality [3] and were especially strongly encouraged to socially isolate early in the pandemic to avoid coronavirus infection. Furthermore, both T2D and CVD have been bidirectionally related to poor mental health [15], so people with CMDs may experience the negative impacts of social isolation especially acutely. Moreover, these individuals may be particularly worried about contracting COVID-19 given their pre-existing health conditions. However, to our knowledge, no previous study has explored mental well-being in relation to social isolation among the older adults with

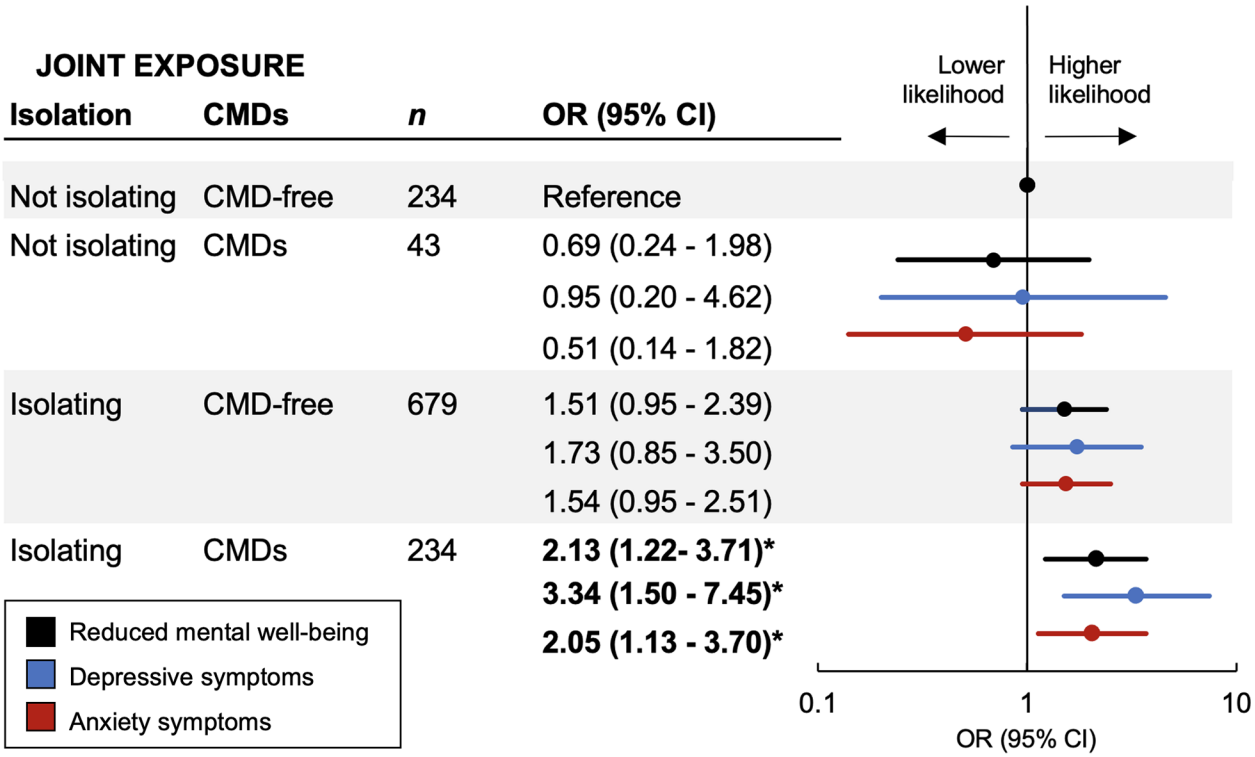

Figure 1. Joint effect of social isolation and cardiometabolic diseases (CMDs) on mental well-being during the first wave of the COVID-19 pandemic. Odds ratios (95% CIs) of reduced mental well-being, depressive symptoms, and anxiety symptoms from logistic regression models adjusted for baseline age, gender, education, living status, smoking status, alcohol consumption, and pre-pandemic depressive symptoms. Interaction between social isolation status and CMD status on reduced mental well-being: *P* for multiplicative interaction = 0.208; *P* for additive interaction = 0.02.

Table 3. Relationship between social isolation and mental well-being among older adults during the first wave of the COVID-19 pandemic, stratified by gender.

		Men			Women		
		n	OR (95% CI)*	*P*-value	*n*	OR (95% CI)*	*P*-value
Social isolation							
No		109	Reference		168	Reference	
Yes		320	1.84 (0.82 – 4.16)	0.139	593	1.80 (1.09 – 2.96)	0.021
Joint exposure†							
Isolation	**CMDs**						
No	No	85	Reference		149	Reference	
No	Yes	24	0.44 (0.08 – 2.58)	0.364	19	0.75 (0.19 – 2.96)	0.683
Yes	No	213	1.39 (0.54 – 3.55)	0.492	466	1.62 (0.95 – 2.77)	0.077
Yes	Yes	107	1.74 (0.61 – 4.95)	0.302	127	2.26 (1.16 – 4.42)	0.017

*Logistic regression models adjusted for baseline age, education, living status, smoking status, alcohol consumption, and pre-pandemic depressive symptoms.

†*P* for multiplicative interaction: 0.280 for men, 0.400 for women; *P* for additive interaction: 0.38 for men, 0.09 for women.

CMDs. We found that people with CMDs were particularly vulnerable to the negative mental health impacts of social isolation. In comparison to people who were CMD-free and not isolating, people with CMDs who isolated showed a 3-fold greater odds ratio for depressive symptoms and 2-fold greater odds ratio for anxiety symptoms.

The health consequences of social isolation for people with CMDs may be even more severe if social isolation itself impacts individuals' ability to self-manage CMDs. Social isolation has been identified as a risk factor for coronary heart disease and stroke [16]. Furthermore, several studies have linked social isolation during the pandemic to reduced levels of physical activity among older adults [17–20], but additional research is needed to determine whether this translates to a worsening of glycemic control or the severity of cardiovascular disease.

A previous investigation indicated that loneliness, anxiety, and insomnia during the pandemic were particularly pronounced among women aged ≥60 years [11]. Consistent with this, our results indicate that the association between social isolation, CMDs, and reduced mental well-being is significant among women but not men. However, this might be due to a smaller sample size of men rather than a gender-specific effect of social isolation. Therefore, the potential greater susceptibility of women to the negative impacts of social isolation on mental well-being warrants further investigation in large population-based studies.

It is possible that the association between social isolation and reduced mental well-being was impacted by acute or time-limited anxiety deriving directly from the pandemic situation. We addressed this in sensitivity analyses stratified by participants' self-reported levels of worry that they themselves (Supplementary Table 1) or a member of their family (Supplementary Table 2) would be affected by COVID-19. In both analyses, the association between social isolation and reduced mental well-being was numerically lower among participants with lower as opposed to higher levels of worry, though neither the less-worried nor the more-worried participants showed a statistically significant association between social isolation and reduced mental well-being, perhaps owing to the small sample size after stratification. By contrast, in a third sensitivity analysis stratified by participants' self-reported feelings of nervousness and stress during the pandemic, social isolation was associated with twice the likelihood (OR, 95% CI) of reduced mental well-being among individuals with low levels of nervousness and stress (2.14, 1.10-4.15), but not those with high levels of nervousness and stress (0.97, 0.48-1.96). Supplementary Table 3 together these sensitivity analyses indicate that acute psychological reactions to the pandemic may have impacted our findings, but their role is complex and difficult to understand in the absence of longitudinal data on mental well-being over the course of the pandemic.

Strengths of this study include the use of a study sample from a well-characterized population-based study, SNAC-K, and the use of a questionnaire developed by a multidisciplinary team of experts. However, some limitations should be acknowledged. First, these findings are based on self-reported information and therefore may be affected by recall bias. Second, given the pandemic restrictions, participants' mental well-being was

measured in terms of self-reported feelings of anxiety and depression via a telephone interview, rather than a formal diagnosis of anxiety or depression based on a clinician's visit. Third, in this study, data on mental well-being was not collected in the same way before and after the onset of the COVID-19 pandemic, thus we could only examine the cross-sectional relationship between social isolation and feelings of depression and anxiety. However, considering the possible influence of pre-pandemic mental health on mental well-being during the first wave of COVID-19, we included participants' MADRS scores from the most recent regular SNAC-K follow-up visit (conducted from 2016 to 2019). Further population-based longitudinal studies are warranted to confirm the social isolation-mental wellbeing association. Additionally, the generalizability of these findings – which reflect an affluent, highly educated, urban population – may be limited, particularly given the voluntary nature of Sweden's COVID-19 prevention recommendations, which differed substantially from the mandates issued in most other western countries. Finally, we could not rule out the influence of potential residual confounding due to unmeasured factors, like the number of social contacts or the severity of CMDs, which may be related to both mental health and the choice to self-isolate.

To the best of our knowledge, this is the first study to examine the combined impact of social isolation and CMDs on mental well-being during the COVID-19 pandemic. Pandemic-related social isolation was associated with depressive and anxiety symptoms in older adults, particularly those with cardiometabolic comorbidities. Our findings highlight the collateral damage of social isolation as a COVID-19 prevention strategy and underscore the need for mental health support for older adults during subsequent waves of the current pandemic. Furthermore, in the unfortunate but highly probable event of a future pandemic on the scale of COVID-19 [14], the amplified effects of social isolation on people with CMDs should be kept in mind and addressed earlier with focused preventive strategies. Together with reports from other geographical settings, the findings from our study may contribute to an open, cautious, and impartial discussion about what countries have gained and lost as a consequence of the exceptional public health measures (both mandated and voluntary) related to the COVID-19 pandemic.

MATERIALS AND METHODS

Study population

The study population was derived from SNAC-K, an ongoing population-based cohort study that includes a random sample of older adults aged ≥60 years living in central Stockholm, Sweden. Between May and September 2020, individuals who participated in the regular SNAC-K follow-up assessment in 2016-2019 were invited to participate in a structured survey administered over the telephone by trained SNAC-K staff to assess their behaviors and direct and indirect health consequences during the first wave of the COVID-19 pandemic (i.e., since March 2020). The telephone questionnaire was developed by the SNAC-K data collection team with input from experts in geriatric medicine, mental health, neurology, and public health. The questions contained some items from the regular SNAC-K assessments (which participants undergo every 3 or 6 years) as well as items from the WHO Europe survey tool [21]. Before the interview, SNAC-K staff explained to participants that all questions referred specifically to the pandemic context. People with a known diagnosis of dementia, very impaired hearing, and those who were living in care or nursing homes were excluded from the telephone questionnaire. Of 1,231 participants who completed the telephone questionnaire (91.9% response rate), we excluded 25 with missing information on pandemic-related mental well-being (i.e., depressive and anxiety symptoms) and 16 with missing information on social isolation, leaving a total of 1,190 participants for the current study (Supplementary Figure 1).

The study was approved by the Karolinska Institutet Ethical Committee and the Regional Ethical Review Board in Stockholm, Sweden. All participants provided informed and written consent.

Data collection

The SNAC-K protocol has been described in detail previously [22]. Briefly, at each wave of follow-up, trained nurses and physicians collected data on demographic factors, lifestyle factors, medication use, and medical history. Additionally, blood samples were collected for laboratory tests (e.g., glycated hemoglobin).

Education level was defined as elementary, high school, or university. Smoking status was grouped into never, former, or current smokers. Alcohol consumption was categorized as no/occasional, light-to-moderate (1–14 drinks/week for men or 1–7 drinks/week for women), or heavy (>14 drinks/week for men or >7 drinks/week for women) drinking [23]. Living status was dichotomized as living alone or not living alone (including living with a partner, children or grandchildren, siblings or friends, etc.). Pre-pandemic levels of depressive symptoms were defined as participants' Montgomery-Asberg Depression Rating Scale (MADRS) score during the most recent regular SNAC-K follow-up assessment (conducted from 2016 to 2019) [24, 25]. Information on medical history collected from the Swedish National Patient Registry

(NPR) using codes from the International Classification of Disease, 10th version (ICD-10).

Assessment of social isolation

In the telephone questionnaire, participants were asked to report whether or not they had been staying at home and avoiding in-person social contact to minimize their chances of being infected by the coronavirus. Social isolation status was dichotomized as isolating vs. not isolating.

Assessment of mental well-being

The telephone questionnaire included questions to assess mental well-being since the onset of the COVID-19 pandemic in Sweden in March 2020. Participants' experience of depression during the pandemic was assessed by the question, "Have you experienced depressive symptoms since March 2020?" Participants responded using a 1 to 6 scale, with 1 indicating a "neutral mood" and 6 indicating "consistent experience of maximum depression." Participants were coded as having experienced depressive symptoms if they scored 3 (i.e., "predominant experience of depression, but brighter moments occur") or higher. Participants' experience of anxiety during the pandemic was ascertained by two questions: 1) "Have you experienced feelings of anxiety since March 2020?" (ranging from 1, "mostly calm," to 6, "prolonged panic attacks; overwhelming feelings of fear that cannot be overcome on their own"); and "Have you experienced anxiety symptoms since March 2020?" (ranging from 1, "no excessive anxiety," to 6, "disabling anxiety; constant brooding over small things, calming assurances have no effect"). Participants were coded as having experienced anxiety if they scored 3 or higher on either question. We additionally created a combined mental well-being endpoint defined as reduced mental well-being (i.e., experience of either depression or anxiety) or sound mental well-being (i.e., experience of neither depression nor anxiety).

Assessment of CMDs

CMD status, defined as the presence of T2D and/or CVD [26], was assessed at the latest regular SNAC-K follow-up visit using data from multiple sources. T2D was ascertained based on self-reported medical history, glucose-lowering medication use, medical records from the NPR (ICD-10 code E11), or glycated hemoglobin ≥6.5% [27]. CVD was identified based on self-reported medical history or medical records from the NPR (including ischemic heart disease [ICD-10 codes: I20-22, I24-25, Z951, and Z955], atrial fibrillation [code I48], heart failure [I110, I130, I132, I27, I280, I42–43, I50, I515, I517, I528, Z941, and Z943], cerebrovascular disease [G45-46, I60-64, I67, and I69], or other cardiovascular diseases [I09, I281, I310-311, I456, I495, I498, I70-72, I790-791, I950-951, I958, Q20-21, Q24-28, and Z958-959]).

Statistical analyses

Characteristics of socially isolating vs. not isolating participants were compared using Chi-square tests for categorical variables and t tests for continuous variables. Data were presented as numbers and percentages for categorical variables and as means and standard deviations for continuous variables.

Odds ratios and 95% confidence intervals for the association between social isolation status and depression, anxiety, or overall reduced mental well-being were obtained from logistic regression analyses. Models were adjusted for age, gender, education, living status, smoking status, alcohol consumption, and pre-pandemic levels of depressive symptoms (i.e. MADRS score from the most recent regular SNAC-K follow-up visit).

We additionally assessed the joint effect of isolation status and CMDs on mental well-being. Participants were categorized into four groups according to combined social isolation (yes vs. no) and CMD (CMD-free vs. any CMD) status. We assessed the additive interaction between social isolation status and CMDs using the attributable proportion due to interaction (AP). We examined the multiplicative interaction between social isolation status and CMDs by adding the cross-product term (social isolation status × CMD status) into the model. To assess possible gender-specific aspects of the associations between social isolation, CMDs, and mental well-being, we repeated all analyses separately among men and women.

Finally, we accounted for potential acute or time-limited anxiety deriving directly from the pandemic situation in sensitivity analyses stratified by participants' level of worry about being affected by COVID-19 ("not at all," "somewhat," or "moderately" vs. "very" or "extremely"), level of worry about family members being affected by COVID-19 ("not at all," "somewhat," or "moderately" vs. "very" or "extremely"), and feelings of nervousness and stress ("never," "rarely," or "sometimes" vs. "quite often" or "very often") since the beginning of the pandemic.

All *P*-values were two-sided, and *P*-values <0.05 were considered statistically significant. Statistical analyses were performed using Stata SE 16.0 (StataCorp, College Station, TX).

Abbreviations

CI: confidence interval; CMDs: cardiometabolic diseases; COVID-19: coronavirus disease 2019; CVD: cardiovascular disease; ICD-10: International Classification of Disease, 10th version; MADRS: Montgomery-Asberg Depression Rating Scale; NPR: National Patient Registry; OR: odds ratio; SNAC-K: Swedish National Study on Aging and Care in Kungsholmen; T2D: type 2 diabetes.

AUTHOR CONTRIBUTIONS

A.D., J.G., W.X., D.L.V, and A.C.L. contributed to the conception and design of the study. A.D. and J.G. performed the statistical analyses, conducted the literature search, and drafted the first version of the manuscript. W.X., D.L.V, A.C.L, and L.F. reviewed and edited the first version of the manuscript. L.F. designed, initiated, and directed SNAC-K. All authors critically revised the manuscript for important intellectual content. All authors made a significant contribution to finalize the manuscript and approved the final version for publication. A.D. and J.G. are the guarantors of this work and, as such, have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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REFERENCES

1. WHO Director-General's opening remarks at the media briefing on COVID-19. 2020. https://www.who.int/director-general/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020

2. Bauer P, Brugger J, König F, Posch M. An international comparison of age and sex dependency of COVID-19 deaths in 2020: a descriptive analysis. Sci Rep. 2021; 11:19143. https://doi.org/10.1038/s41598-021-97711-8 PMID:34580322

3. Dessie ZG, Zewotir T. Mortality-related risk factors of COVID-19: a systematic review and meta-analysis of 42 studies and 423,117 patients. BMC Infect Dis. 2021; 21:855. https://doi.org/10.1186/s12879-021-06536-3 PMID:34418980

4. Vetrano DL, Tazzeo C, Palmieri L, Marengoni A, Zucchelli A, Lo Noce C, Onder G, and Italian National Institute of Health Covid-Mortality Group. Comorbidity status of deceased COVID-19 in-patients in Italy. Aging Clin Exp Res. 2021; 33:2361–5. https://doi.org/10.1007/s40520-021-01914-y PMID:34169447

5. The Public Health Agency of Sweden. National general guidelines to reduce the spread of COVID-19. https://www.folkhalsomyndigheten.se/the-public-health-agency-of-sweden/communicable-disease-control/covid-19/national-general-guidelines-to-reduce-the-spread-of-covid-19/

6. National Academies of Sciences, Engineering, and Medicine, and Division of Behavioral and Social Sciences and Education, and Health and Medicine Division, and Board on Behavioral, Cognitive, and Sensory Sciences, and Board on Health Sciences Policy, and Committee on the Health and Medical Dimensions of Social Isolation and Loneliness in Older Adults. Social Isolation and Loneliness in Older Adults: Opportunities for the Health Care System. Washington (DC): National Academies Press (US). 2020. https://doi.org/10.17226/25663

7. Santini ZI, Jose PE, York Cornwell E, Koyanagi A, Nielsen L, Hinrichsen C, Meilstrup C, Madsen KR, Koushede V. Social disconnectedness, perceived isolation, and symptoms of depression and anxiety among older Americans (NSHAP): a longitudinal mediation analysis. Lancet Public Health. 2020; 5:e62–70. https://doi.org/10.1016/S2468-2667(19)30230-0

PMID:31910981

8. Zhao SZ, Wong JY, Wu Y, Choi EP, Wang MP, Lam TH. Social Distancing Compliance under COVID-19 Pandemic and Mental Health Impacts: A Population-Based Study. Int J Environ Res Public Health. 2020; 17:6692. https://doi.org/10.3390/ijerph17186692 PMID:32937929

9. Gloster AT, Lamnisos D, Lubenko J, Presti G, Squatrito V, Constantinou M, Nicolaou C, Papacostas S, Aydın G, Chong YY, Chien WT, Cheng HY, Ruiz FJ, et al. Impact of COVID-19 pandemic on mental health: An international study. PLoS One. 2020; 15:e0244809. https://doi.org/10.1371/journal.pone.0244809 PMID:33382859

10. COVID-19 Mental Disorders Collaborators. Global prevalence and burden of depressive and anxiety disorders in 204 countries and territories in 2020 due to the COVID-19 pandemic. Lancet. 2021; 398:1700–12. https://doi.org/10.1016/S0140-6736(21)02143-7 PMID:34634250

11. Wong SY, Zhang D, Sit RW, Yip BH, Chung RY, Wong CK, Chan DC, Sun W, Kwok KO, Mercer SW. Impact of COVID-19 on loneliness, mental health, and health service utilisation: a prospective cohort study of older adults with multimorbidity in primary care. Br J Gen Pract. 2020; 70:e817–24. https://doi.org/10.3399/bjgp20X713021 PMID:32988955

12. Sepúlveda-Loyola W, Rodríguez-Sánchez I, Pérez-Rodríguez P, Ganz F, Torralba R, Oliveira DV, Rodríguez-Mañas L. Impact of Social Isolation Due to COVID-19 on Health in Older People: Mental and Physical Effects and Recommendations. J Nutr Health Aging. 2020; 24:938–47. https://doi.org/10.1007/s12603-020-1469-2 PMID:33155618

13. Villani ER, Vetrano DL, Damiano C, Paola AD, Ulgiati AM, Martin L, Hirdes JP, Fratiglioni L, Bernabei R, Onder G, Carfì A. Impact of COVID-19-Related Lockdown on Psychosocial, Cognitive, and Functional Well-Being in Adults With Down Syndrome. Front Psychiatry. 2020; 11:578686. https://doi.org/10.3389/fpsyt.2020.578686 PMID:33192717

14. Marani M, Katul GG, Pan WK, Parolari AJ. Intensity and frequency of extreme novel epidemics. Proc Natl Acad Sci USA. 2021; 118:e2105482118. https://doi.org/10.1073/pnas.2105482118 PMID:34426498

15. Pan A, Lucas M, Sun Q, van Dam RM, Franco OH, Manson JE, Willett WC, Ascherio A, Hu FB. Bidirectional association between depression and type 2 diabetes mellitus in women. Arch Intern Med. 2010; 170:1884–91. https://doi.org/10.1001/archinternmed.2010.356 PMID:21098346

16. Valtorta NK, Kanaan M, Gilbody S, Ronzi S, Hanratty B. Loneliness and social isolation as risk factors for coronary heart disease and stroke: systematic review and meta-analysis of longitudinal observational studies. Heart. 2016; 102:1009–16. https://doi.org/10.1136/heartjnl-2015-308790 PMID:27091846

17. Mazo GZ, Fank F, Franco PS, Capanema BD, Pereira FD. Impact of Social Isolation on Physical Activity and Factors Associated With Sedentary Behavior in Older Adults During the COVID-19 Pandemic. J Aging Phys Act. 2022; 30:148–52. https://doi.org/10.1123/japa.2020-0456 PMID:34388700

18. Yamada M, Kimura Y, Ishiyama D, Otobe Y, Suzuki M, Koyama S, Kikuchi T, Kusumi H, Arai H. The Influence of the COVID-19 Pandemic on Physical Activity and New Incidence of Frailty among Initially Non-Frail Older Adults in Japan: A Follow-Up Online Survey. J Nutr Health Aging. 2021; 25:751–6. https://doi.org/10.1007/s12603-021-1634-2 PMID:34179929

19. Browne RA, Macêdo GA, Cabral LL, Oliveira GT, Vivas A, Fontes EB, Elsangedy HM, Costa EC. Initial impact of the COVID-19 pandemic on physical activity and sedentary behavior in hypertensive older adults: An accelerometer-based analysis. Exp Gerontol. 2020; 142:111121. https://doi.org/10.1016/j.exger.2020.111121 PMID:33132145

20. Schlichtiger J, Steffen J, Huber BC, Brunner S. Physical activity during COVID-19 lockdown in older adults. J Sports Med Phys Fitness. 2021; 61:164–6. https://doi.org/10.23736/S0022-4707.20.11726-2 PMID:33000934

21. World Health Organization Regional Office for Europe. Survey tool and guidance: rapid, simple, flexible behavioural insights on COVID-19. 2020. https://apps.who.int/iris/handle/10665/333549

22. Lagergren M, Fratiglioni L, Hallberg IR, Berglund J, Elmståhl S, Hagberg B, Holst G, Rennemark M, Sjölund BM, Thorslund M, Wiberg I, Winblad B, Wimo A. A longitudinal study integrating population, care and social services data. The Swedish National study on Aging and Care (SNAC). Aging Clin Exp Res. 2004; 16:158–68. https://doi.org/10.1007/BF03324546 PMID:15195992

23. Wang R, Fratiglioni L, Laveskog A, Kalpouzos G, Ehrenkrona CH, Zhang Y, Bronge L, Wahlund LO, Bäckman L, Qiu C. Do cardiovascular risk factors explain the link between white matter hyperintensities and brain volumes in old age? A population-based study. Eur J Neurol. 2014; 21:1076–82. https://doi.org/10.1111/ene.12319 PMID:24313901

24. Montgomery SA, Asberg M. A new depression scale designed to be sensitive to change. Br J Psychiatry. 1979; 134:382–9. https://doi.org/10.1192/bjp.134.4.382 PMID:444788

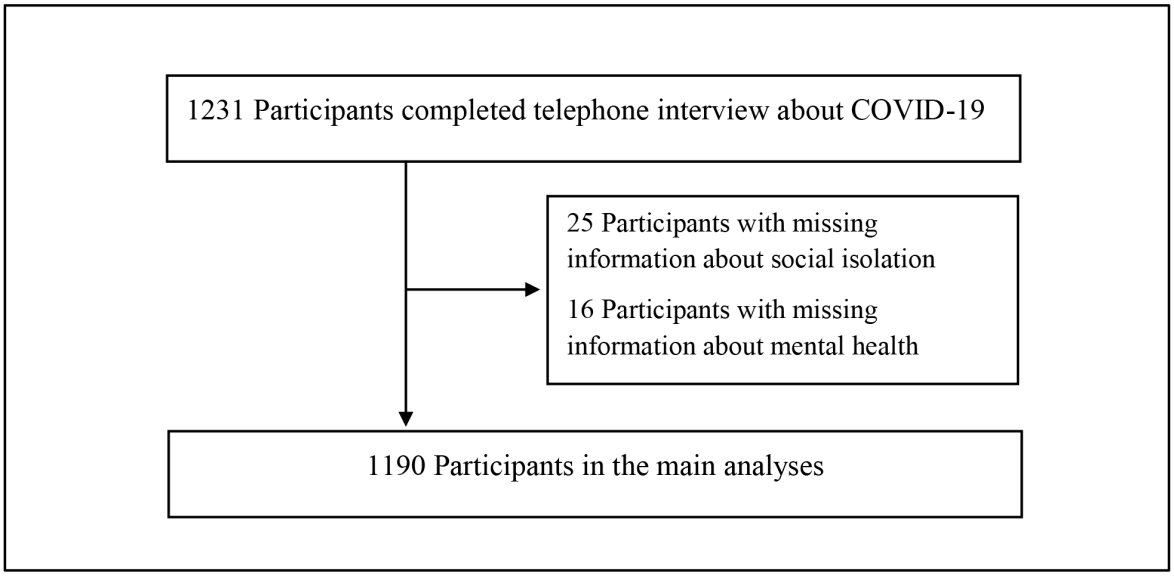
25. Snaith RP, Harrop FM, Newby DA, Teale C. Grade scores of the Montgomery-Asberg Depression and the Clinical Anxiety Scales. Br J Psychiatry. 1986; 148:599–601. https://doi.org/10.1192/bjp.148.5.599 PMID:3779233

26. Di Angelantonio E, Kaptoge S, Wormser D, Willeit P, Butterworth AS, Bansal N, O'Keeffe LM, Gao P, Wood AM, Burgess S, Freitag DF, Pennells L, Peters SA, et al, and Emerging Risk Factors Collaboration. Association of Cardiometabolic Multimorbidity With Mortality. JAMA. 2015; 314:52–60. https://doi.org/10.1001/jama.2015.7008 PMID:26151266

27. American Diabetes Association. Classification and diagnosis of diabetes: Standards of medical care in diabetes. Diabetes Care. American Diabetes Association Inc. 2019; 42: S13–28.

SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Flow chart of the study population.

Supplementary Tables

Supplementary Table 1. Relationship between social isolation and mental well-being among older adults during the first wave of the COVID-19 pandemic, stratified by level of worry about being affected by COVID-19.

		Are you worried about being affected by COVID-19?			
		Not at all / Somewhat / Moderately		Very / Extremely	
		n	OR (95% CI)*	*n*	OR (95% CI)*
Social isolation					
No		254	Reference	20	Reference
Yes		750	1.33 (0.83 – 2.14)	153	1.65 (0.44 – 6.19)
Joint exposure					
Isolation	**CMDs**				
No	No	211	Reference	20	Reference
No	Yes	43	0.81 (0.28 – 2.35)	0	--
Yes	No	560	1.17 (0.70 – 1.98)	113	1.80 (0.47 – 6.90)
Yes	Yes	190	1.71 (0.89 – 3.27)	40	1.26 (0.28 – 5.80)

*Logistic regression models adjusted for baseline age, sex, education, living status, smoking status, alcohol consumption, and pre-pandemic depressive symptoms.

Supplementary Table 2. Relationship between social isolation and mental well-being among older adults during the first wave of the COVID-19 pandemic, stratified by level of worry about family members being affected by COVID-19.

		Are you worried that someone in your family will be affected by COVID-19?			
		Not at all / Somewhat / Moderately		Very / Extremely	
		n	OR (95% CI)*	*n*	OR (95% CI)*
Social isolation					
No		232	Reference	42	Reference
Yes		651	1.39 (0.84 – 2.31)	251	1.74 (0.73 – 4.13)
Joint exposure					
Isolation	**CMDs**				
No	No	196	Reference	36	Reference
No	Yes	36	0.83 (0.25 – 2.71)	6	0.30 (0.3 – 3.35)
Yes	No	478	1.21 (0.69 – 2.12)	193	1.36 (0.53 – 3.48)
Yes	Yes	173	1.81 (0.92 – 3.57)	58	1.86 (0.60 – 5.83)

*Logistic regression models adjusted for baseline age, sex, education, living status, smoking status, alcohol consumption, and pre-pandemic depressive symptoms.

Supplementary Table 3. Relationship between social isolation and mental well-being among older adults during the first wave of the COVID-19 pandemic, stratified by level of nervousness and stress during the first wave of the pandemic.

		How often have you felt nervous or stressed since March 2020?			
		Never / Rarely / Sometimes		Quite often / Very often	
		n	OR (95% CI)*	*n*	OR (95% CI)*
Social isolation					
No		216	Reference	59	Reference
Yes		648	**2.14 (1.10 – 4.15)**	226	0.97 (0.48 – 1.96)
Joint exposure					
Isolation	**CMDs**				
No	No	179	Reference	53	Reference
No	Yes	37	0.90 (0.18 – 4.40)	6	0.64 (0.10 – 4.07)
Yes	No	476	1.91 (0.92 – 3.99)	175	0.87 (0.41 – 1.84)
Yes	Yes	172	2.75 (1.16 – 6.51)	51	1.16 (0.43 – 3.15)

*Logistic regression models adjusted for baseline age, sex, education, living status, smoking status, alcohol consumption, and pre-pandemic depressive symptoms.