Research Paper

Epigenetic age and lung cancer risk in the CLUE II prospective cohort study

Dominique S. Michaud[1], Mei Chung[1,2], Naisi Zhao[1], Devin C. Koestler[3,4], Jiayun Lu[5], Elizabeth A. Platz[5,6], Karl T. Kelsey[7,8]

[1]Department of Public Health and Community Medicine, Tufts University School of Medicine, Tufts University, Boston, MA 02111, USA
[2]Division of Nutrition Epidemiology and Data Science, Friedman School of Nutrition, Tufts University, Boston, MA 02111, USA
[3]Department of Biostatistics and Data Science, University of Kansas Medical Center, Kansas City, KS 66160, USA
[4]University of Kansas Cancer Center, Kansas City, KS 66160, USA
[5]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA
[6]The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD 21231, USA
[7]Department of Epidemiology, Brown University, Providence, RI 02903, USA
[8]Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02903, USA

Correspondence to: Dominique S. Michaud; **email:** Dominique.Michaud@tufts.edu
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ABSTRACT

Background: Epigenetic age, a robust marker of biological aging, has been associated with obesity, low-grade inflammation and metabolic diseases. However, few studies have examined associations between different epigenetic age measures and risk of lung cancer, despite great interest in finding biomarkers to assist in risk stratification for lung cancer screening.
Methods: A nested case-control study of lung cancer from the CLUE II cohort study was conducted using incidence density sampling with 1:1 matching of controls to lung cancer cases (*n* = 208 matched pairs). Prediagnostic blood samples were collected in 1989 (CLUE II study baseline) and stored at −70°C. DNA was extracted from buffy coat and DNA methylation levels were measured using Illumina MethylationEPIC BeadChip Arrays. Three epigenetic age acceleration (i.e., biological age is greater than chronological age) measurements (Horvath, Hannum and PhenoAge) were examined in relation to lung cancer risk using conditional logistic regression.
Results: We did not observe associations between the three epigenetic age acceleration measurements and risk of lung cancer overall; however, inverse associations for the two Hannum age acceleration measures (intrinsic and extrinsic) were observed in men and among younger participants, but not in women or older participants. We did not observe effect modification by time from blood draw to diagnosis.
Conclusion: Findings from this study do not support a positive association between three different biological age acceleration measures and risk of lung cancer. Additional studies are needed to address whether epigenetic age is associated with lung cancer in never smokers.

INTRODUCTION

Lung cancer remains the leading cause of cancer deaths in the US and worldwide [1]. Substantial effort has been devoted to identifying heritable genomic markers that could aid in classification of high-risk individuals for screening purposes [2, 3]. While results from these studies are promising, predictive modeling using

genomic markers (in addition to age and smoking) are currently not sufficiently discriminatory or calibrated to be useful in clinical settings for risk prediction. Identifying high risk groups could improve efficiency in lung cancer screening (with low-dose computed tomography) and reduce racial inequalities associated with the current recommendations for screening based on smoking history [4]. Thus, there is an urgent need to identify biomarkers that can reflect biological processes in lung cancer development and that could, eventually, be incorporated into models for risk stratification.

Variation in DNA methylation levels in peripheral blood leukocytes reflect genetic imprinting, environmental exposures, and the lineage differentiation that gives rise to immune cell subtypes [5]. Recent studies using epigenetic markers in blood have identified differentially methylated regions in smokers [6, 7]; DNA methylation levels in these regions remained strongly associated with lung cancer mortality after adjusting for smoking history [6]. Epigenetic aging measures or "clocks" have also been developed to reflect biological age in tissue and blood [8]. These epigenetic clocks are highly correlated with chronological age, but can deviate from chronological age, reflecting changes in immunity and cellular senescence, which are closely aligned with health and disease. Epigenetic age acceleration is the difference between the predicted biological age (based on the epigenetic measurements) and the given chronological age. Recent studies have linked epigenetic age acceleration to a range of disease outcomes, including all-cause mortality [9, 10], cardiovascular disease (CVD) incidence [11], coronary heart disease (CHD) mortality [12], cancer incidence [13] and cancer mortality [12]. Epigenetic age acceleration estimated using the Horvath and Hannum clocks, known as "first generation clocks", is highly heritable (~0.4 [9]) and has been associated with CVD and cancer risk factors, including obesity, low-grade inflammation [14], and metabolic syndrome [15]. The newer generation of clocks, such as PhenoAge clock, have been developed based on associations with age, all-cause mortality, and several clinical biomarkers [16].

To date, studies evaluating epigenetic age acceleration and lung cancer risk have been inconsistent. The first nested case-control study conducted in the Women's Health Initiative (WHI) observed a strong positive association [17], while a larger nested case-control study (Melbourne Collaborative Consortium Study; MCCS) reported no associations for the Horvath and Hannum clocks [18] but positive association with PhenoAge clock [13]. Additionally, stratified analyses by time since blood drawn were performed in the MCCS, and the results showed no significant differences in the positive associations between PhenoAge acceleration and lung cancer risk by time since blood drawn (≤5 years, 5–10 years, or >10 years) [13]. In the WHI study, the positive associations were stronger among women developing lung cancer at 70 or more years and among current smokers. In the MCCS [18], men and women were combined, and no stratified analyses were conducted by sex, to inform whether the association was restricted to women.

It is important to examine whether epigenetic age is associated with lung cancer risk across multiple prospective studies to determine its utility as a potential biomarker to be considered for risk stratification in the selection of high-risk individuals for lung cancer screening. Thus, for this analysis, we conducted a nested case-control analysis of 208 lung cancer cases and 208 matched controls with archived pre-diagnostic blood samples (from 1989). The case-control study is nested in the CLUE II cohort study, a predominantly White cohort of men and women, based in Maryland, USA.

METHODS

Study population

Individuals included in this analysis were selected from participants in the CLUE II study, a prospective cohort study initiated in Washington County, Maryland, in 1989 [19, 20]. The CLUE II study was an outgrowth of a previous study (CLUE I) that had been conducted in the same region in 1974. Some of the participants in CLUE II had been participants in CLUE I (about a third), but this was not a requirement for recruitment into the overall CLUE II cohort. At the baseline visit (1989 for CLUE II), brief medical histories, blood pressure readings, and blood samples were collected on 32,894 participants (25,076 of which were residents of Washington County). Mobile office trailers were used to recruit participants and to collect blood samples. Blood was drawn into 20 ml heparinized Vacutainers (Becton- Dickinson, Rutherford, NJ), kept at 4°C until the plasma was separated, usually within 2–6 h, and divided into aliquots of plasma, buffy coat, and red blood cells. All samples are stored at −70°C. Comparisons with published figures from the 1990 Census indicated that approximately 30 percent of adult residents had participated: 98.3% were White, reflecting the population of this county, and 59% were female, with the better-educated and the age group 45 to 70 years having higher participation rates. Self-reported attained education, weight and height, cigarette smoking status, number of cigarettes smoked per day, and cigar/pipe smoking status were recorded for each participant at baseline.

The Institutional Review Board at the Johns Hopkins Bloomberg School of Public Health and the Tufts

University Health Sciences Campus Institutional Review Board approved this study.

Lung cancer cases and matched controls

Incident lung cancer cases were ascertained from linkage to the Washington Co. Cancer Registry (1989-January 2018) and the Maryland Cancer Registry (1992-January 2018). The Maryland Cancer Registry is certified by the North American Association of Central Cancer Registries as being more than 95% complete. Compared with the Maryland Cancer Registry, the Washington County Cancer Registry captured 98% of the lung cancer cases diagnosed in Washington County residents in 1998. Cancer deaths were identified from state vital statistics, next of kin, and obituaries and confirmed on death certificates; underlying cause of death was obtained from the death certificates. Between 1989 and January 2018, a total of 241 eligible incident first primary lung cancer cases were ascertained from CLUE II participants with blood samples, and who had also previously participated in CLUE I (a requirement based on a shared study population). All 241 lung cancer cases (ICD 9 162 and ICD10 C34) were confirmed by pathology report.

Controls were selected from among CLUE II participants who had also participated in CLUE I. Matching was conducted using incidence density sampling such that a control had to be alive and free of cancer at the time the matched case was diagnosed with lung cancer. One control was matched to each case on the following factors: age (±3 year), sex, race, cigarette smoking status and intensity, cigar/pipe smoking status, and date of blood draw (±4 months). Controls who later became cases were also included as cases with their new matched controls.

DNA methylation measurements

Extracted DNA was bisulfite-treated using the EZ DNA Methylation Kit (Zymo), and DNA methylation was measured with the 850K Illumina Infinium MethylationEPIC BeadChip Arrays (Illumina, Inc, CA, USA). All samples and all array measurements were performed blinded to case-control status. Details on DNA methylation measurements, data preprocessing processing and quality control assessment/screening have been published [21]. Due to lack of remaining DNA, 8 of the 241 incident cases were removed from the dataset before matching.

Estimation of peripheral blood leukocyte composition

Peripheral blood leukocyte subtypes proportions were estimated using a newly expanded reference-based deconvolution library EPIC IDOL-Ext [22]. This library used the IDOL methodology [23] to optimize the currently available six-cell reference library [24] to deconvolute the proportions of 12 leukocyte subtypes in peripheral blood (neutrophils, eosinophils, basophils, monocytes, naïve and memory B cells, naïve and memory CD4+ and CD8+ cells, natural killer, and T regulatory cells).

Data processing

All methylation data preprocessing and normalization steps were performed using the Bioconductor packages. The raw IDAT files from methylation array were processed using the minfi Bioconductor package [25, 26]. Within-array correction for background fluorescence and dye-biases were performed using the Noob methodology via the function "preprocessNoob" in the minfi Bioconductor package [27]. The QCinfo function in ENmix Bioconductor [28] package was then used to identify and remove poor quality samples and probes. Samples were excluded if: 1) more than 5% of probes had quality issues as addressed using the detection *p*-value, 2) the bisulfite conversion intensity was lower than 3 standard deviations from the mean, or 3) the mean average intensity and/or the mean average beta values were more than 3 times IQR from the upper quartile or less than 3 time IQR from the lower quartile of the average intensity values or beta value across the samples. In addition, we excluded probes that had detection *p*-values exceeding 1×10^{-6} (compared to the negative background probes) in more than 5% of the samples. After sample- and probe- level quality control, we corrected the type II probe bias to make the methylation distribution of type II feature comparable to the distribution of type I feature using the beta mixture quantile dilation intra-sample normalization method [29], implemented using "BMIQ" function in the wateRmelon Bioconductor package [30]. Principal components analysis (PCA) was performed on the BMIQ-adjusted values and the top K principal components (K determined using a previously described random matrix theory approach [31]) to detect whether the microarray dataset had the batch effect. Then ctrlsva function in ENmix Bioconductor packages [32] was used to estimate the surrogate variables of batch effects [33]. The estimated surrogate variables were used in downstream analyses to adjust for batch effects and other unwanted technical sources of variation.

Smoking methylation score

A smoking methylation score was calculated to estimate individual pack-years of smoking based on known smoking-related DNA methylation alterations [34]. The

smoking methylation score was first developed to predict smoking pack-years using smoking 'signatures' reported by large-scale epigenome-wide association meta-analyses [34]. The score correlates with gene expression changes affected by smoking and can be utilized in lieu of self-reported smoking data.

Estimation of epigenetic age

Three DNAm clocks (Hannum [35], Horvath [36], and PhenoAge [16]) were used to estimate subjects' DNAm age (using ENMix Biocondontor package: https://rdrr.io/bioc/ENmix/man/methyAge.html). For each of the three DNAm clocks, DNAm age acceleration (AA) was defined by regressing DNAm age on chronologic age and calculating the difference between the observed chronological age and the fitted DNAm age (i.e., the residual). Additionally, intrinsic epigenetic age acceleration (IEAA) metrics were calculated using the residuals from the linear regression fit to DNAm age on chronologic age, adjusted for estimated blood cell composition [37, 24] (for comparability with prior studies, we did not update the reference library for the IEAA measurements). Three subjects with an absolute value of the age acceleration estimate greater than 3 standard deviations (SDs) from the mean were excluded from the regression analyses; sensitivity analyses conducted retaining these 3 subjects did not materially modify the results.

Statistical analyses

Given the 1:1 case-control matching present in our study, conditional logistic regression models were used to examine the association between epigenetic age acceleration and lung cancer risk. As age, sex, and smoking status (never, former, current), smoking intensity (cigarettes/day) and cigar/pipe smoking were matching factors, these were implicitly adjusted for when using conditional regression. In the conditional regression model, we additionally adjusted for BMI as a continuous variable, batch effect (for methylation arrays), and a previously described methylation-predicted variable to capture pack-years smoked [34]. We conducted stratified analyses by sex, median age, lung cancer histology (non-small cell lung cancer [NSCLC], small cell lung cancer [SCLC]), and length of time between blood draw and diagnosis (≤10, >10 years) to evaluate potential effect modification. We did not adjust for methylation-derived cell proportions given that the intrinsic epigenetic age (IEAA) measures already account for immune cells. Pearson's correlation was used to examine correlation between epigenetic age acceleration and methylation predicted immune cell proportions. All statistical analyses were performed in R (version 3.5.1).

Availability of data materials

The data cannot be deposited into a controlled access database due to a State of Maryland law that established the Maryland Cancer Registry (where the lung cancer data was obtained).

RESULTS

The final analysis consisted of 208 cases and matched 208 controls. As a result of matching, lung cancer cases and controls had similar age (mean age: 55.9 years among controls, 58.3 years among cases), sex distribution (54.3% females in cases and controls), smoking status (51% current smokers in cases and controls; 39% former smokers in cases and controls), smoking intensity (25 cigarettes per day in current smokers among cases; 24 cigarettes/day in current smokers among controls) and cigar or pipe smoking (15% ever in cases and controls). Only 3 cases, and no controls, were non-White individuals. Cases and controls were also similar with respect to BMI (mean, in kg/m^2, BMI 26.0 cases, 26.2 controls). Most lung cancer cases were NSCLC (74%). Cases were diagnosed a mean of 14 years post-blood donation (median 14 years; range >0–29 years; all cases were incident cases).

In this population, men, and cases with a shorter time between blood draw and cancer diagnosis, were more likely to have age acceleration (vs. deceleration) in all 3 epigenetic clock measures (Table 1). Other characteristics, including smoking and BMI were very similar for acceleration and deceleration of epigenetic age in all 3 measures.

Overall, we did not observe any associations between the 3 epigenetic age acceleration measures and lung cancer risk using both continuous and categorical variables for age acceleration (Table 2). Associations were similar when stratified by time between blood drawn and cancer diagnosis (Table 3).

Given that in a prior study positive associations were modified by age and smoking status and were only reported for women [17], we conducted stratified analyses by age (<65 years, ≥65 years), smoking status (current vs. former smokers) and sex. There was an inverse trend for the Hannum measurements in men but not in women (Table 4). Associations for all three age acceleration measures were statistically significantly inversely associated with lung cancer in the younger (<65 years) but not older age group (Supplementary Table 1). Associations were similar among current and former smokers after adjusting for methylation predicted pack-years; associations were not estimated among never smokers due to small numbers

Table 1. Baseline characteristics for the CLUE II population, by age acceleration vs. deceleration.

	Overall	AA_Hannum		AA_Horvath		AA_Pheno	
		Acceleration	Deceleration	Acceleration	Deceleration	Acceleration	Deceleration
N	**416**	**210**	**203**	**208**	**205**	**220**	**193**
Case	208 (50.0%)	104 (49.5%)	101 (49.8%)	99 (47.6%)	107 (52.2%)	114 (51.8%)	91 (47.2%)
Control	208 (50.0%)	106 (50.5%)	102 (50.2%)	109 (52.4%)	98 (47.8%)	106 (48.2%)	102 (52.8%)
Age (yrs), mean (SD)	57.1 (10.0)	57.3 (9.3)	56.6 (10.5)	57.1 (8.9)	56.9 (11.0)	56.8 (9.4)	57.2 (10.7)
Female, *n* (%)	226 (54.3%)	90 (42.9%)	133 (65.5%)	97 (46.6%)	127 (62.0%)	108 (49.1%)	115 (59.6%)
Time Difference (yrs), mean (SD)	14.9 (7.9)	13.9 (7.3)	16.2 (8.2)	14.5 (7.6)	15.4 (8.1)	14.7 (7.7)	15.3 (8.2)
Smoking status							
Never	44 (10.6%)	23 (11.0%)	21 (10.3%)	28 (13.5%)	16 (7.8%)	22 (10.0%)	21 (10.9%)
Former	160 (38.5%)	86 (41.0%)	73 (36.0%)	80 (38.5%)	79 (38.5%)	86 (38.6%)	74 (38.3%)
Current	212 (50.9%)	101 (48.0%)	109 (53.7%)	100 (48.0%)	110 (53.7%)	112 (51.4%)	98 (50.8%)
BMI (kg/m^2), mean (SD)	26.1 (4.4)	26.2 (4.3)	26.1 (4.5)	26.7 (4.5)	25.6 (4.2)	26.0 (4.4)	26.2 (4.4)
Normal and underweight	179 (43.0%)	87 (41.4%)	90 (44.3%)	76 (36.5%)	100 (48.8%)	94 (42.3%)	82 (42.5%)
Overweight	171 (41.1%)	89 (42.4%)	81 (39.9%)	91 (43.8%)	80 (39.0%)	95 (43.2%)	76 (39.4%)
Obese	66 (15.9%)	34 (16.2%)	32 (15.8%)	41 (19.7%)	25 (12.2%)	31 (14.4%)	35 (18.1%)

Abbreviations: SD: standard deviation; AA: age acceleration; BMI: body mass index.

Table 2. Odds ratios and 95% confidence intervals for the association between epigenetic age acceleration (using 3 different measures) and the risk of lung cancer in the CLUE II study.

Age acceleration measure	OR (95% CI)*	*p*-value*
AA_Hannum		
Q1	1 (ref.)	
Q2	0.96 (0.55, 1.66)	0.88
Q3	0.74 (0.42, 1.30)	0.29
Q4	0.74 (0.42, 1.31)	0.31
Continuous (per 1 SD)	0.82 (0.64, 1.05)	0.12
AA_Horvath		
Q1	1 (ref.)	
Q2	0.80 (0.46, 1.41)	0.45
Q3	0.65 (0.37, 1.16)	0.15
Q4	0.76 (0.43, 1.33)	0.33
Continuous (per 1 SD)	0.85 (0.68, 1.07)	0.17
AA_Pheno		
Q1	1 (ref.)	
Q2	0.61 (0.33, 1.11)	0.10
Q3	0.79 (0.44, 1.40)	0.42
Q4	0.89 (0.51, 1.56)	0.69
Continuous (per 1 SD)	0.92 (0.76, 1.10)	0.36
IEAA_Hannum		
Q1	1 (ref.)	
Q2	0.74 (0.42, 1.31)	0.30
Q3	0.62 (0.35, 1.10)	0.10
Q4	0.70 (0.40, 1.24)	0.22
Continuous (per 1 SD)	0.87 (0.67, 1.14)	0.33

IEAA_Horvath		
Q1	1 (ref.)	
Q2	0.52 (0.28, 0.94)	0.031
Q3	0.84 (0.48, 1.47)	0.54
Q4	0.82 (0.47, 1.42)	0.47
Continuous (per 1 SD)	0.93 (0.73, 1.19)	0.58
IEAA_Pheno		
Q1	1 (ref.)	
Q2	0.75 (0.42, 1.35)	0.33
Q3	0.92 (0.52, 1.65)	0.79
Q4	0.87 (0.49, 1.55)	0.64
Continuous (per 1 SD)	0.90 (0.74, 1.10)	0.30

Abbreviations: IEAA: Intrinsic epigenetic age acceleration; ref.: referent. *Conditional logistic regression models, adjusting for batch effects, BMI, and smoking predicted pack years (using DNA methylation).

Table 3. Odds ratios and 95% confidence intervals for the association between epigenetic age acceleration (using 3 different measures) and the risk of lung cancer in the CLUE II study, stratified by time between blood draw and cancer diagnosis (in cases; matched date in controls).

	Time difference ≤10 yrs		Time difference >10 yrs	
	OR (95% CI)*	***p*-value***	**OR (95% CI)***	***p*-value***
AA_Hannum				
Q1	1 (ref.)		1 (ref.)	
Q2	1.38 (0.62, 3.07)	0.43	0.79 (0.42, 1.48)	0.46
Q3	1.09 (0.49, 2.43)	0.84	0.61 (0.31, 1.17)	0.14
Q4	0.70 (0.29, 1.66)	0.41	0.77 (0.41, 1.45)	0.41
		p for trend = 0.34		*p* for trend = 0.33
AA_Horvath				
Q1	1 (ref.)		1 (ref.)	
Q2	0.75 (0.33, 1.69)	0.48	0.77 (0.41, 1.46)	0.43
Q3	0.66 (0.28, 1.51)	0.32	0.62 (0.32, 1.20)	0.15
Q4	0.74 (0.33, 1.64)	0.46	0.75 (0.39, 1.41)	0.37
		p for trend = 0.44		*p* for trend = 0.30
AA_Pheno				
Q1	1 (ref.)		1 (ref.)	
Q2	0.84 (0.36, 1.99)	0.69	0.53 (0.26, 1.06)	0.07
Q3	1.35 (0.59, 3.07)	0.48	0.59 (0.30, 1.16)	0.13
Q4	0.67 (0.28, 1.58)	0.36	1.06 (0.56, 1.98)	0.86
		p for trend = 0.54		*p* for trend = 0.69
IEAA_Hannum				
Q1	1 (ref.)		1 (ref.)	
Q2	0.61 (0.26, 1.42)	0.25	0.75 (0.40, 1.41)	0.37
Q3	0.91 (0.42, 1.94)	0.80	0.50 (0.25, 1.00)	0.05
Q4	0.58 (0.26, 1.34)	0.20	0.74 (0.39, 1.42)	0.37
		p for trend = 0.35		*p* for trend = 0.28
IEAA_Horvath				
Q1	1 (ref.)		1 (ref.)	
Q2	0.39 (0.15, 0.97)	0.044	0.58 (0.30, 1.14)	0.11

Q3	0.90 (0.40, 2.01)	0.79	0.81 (0.43, 1.53)	0.52
Q4	0.82 (0.37, 1.80)	0.62	0.77 (0.41, 1.44)	0.41
		p for trend = 0.93		*p* for trend = 0.59
IEAA_Pheno				
Q1	1 (ref.)		1 (ref.)	
Q2	0.84 (0.36, 1.98)	0.70	0.66 (0.34, 1.31)	0.24
Q3	1.27 (0.56, 2.89)	0.57	0.81 (0.41, 1.60)	0.54
Q4	0.59 (0.25, 1.41)	0.24	1.06 (0.55, 2.03)	0.86
		p for trend = 0.33		*p* for trend = 0.67

Abbreviations: IEAA: Intrinsic epigenetic age acceleration; ref.: referent. *Conditional logistic regression models, adjusting for batch effects, BMI, and smoking predicted pack years (using DNA methylation).

Table 4. Odds ratios and 95% confidence intervals for the association between epigenetic age acceleration (using 3 different measures) and the risk of lung cancer in the CLUE II study, stratified by sex.

	Female		Male	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**
AA_Hannum				
Q1	1 (ref.)		1 (ref.)	
Q2	0.74 (0.28, 2.00)	0.56	0.78 (0.30, 2.00)	0.60
Q3	1.18 (0.49, 2.84)	0.71	0.50 (0.21, 1.19)	0.12
Q4	0.79 (0.33, 1.88)	0.60	0.29 (0.10, 0.80)	0.017
		P trend = 0.78		*P* trend =0.015
AA_Horvath				
Q1	1 (ref.)		1 (ref.)	
Q2	0.92 (0.40, 2.13)	0.84	0.72 (0.30, 1.73)	0.47
Q3	0.82 (0.34, 1.99)	0.66	0.51 (0.21, 1.24)	0.14
Q4	0.92 (0.40, 2.15)	0.85	0.50 (0.19, 1.35)	0.17
		P trend = 0.80		*P* trend = 0.13
AA_Pheno				
Q1	1 (ref.)		1 (ref.)	
Q2	0.70 (0.28, 1.76)	0.44	1.11 (0.43, 2.86)	0.83
Q3	0.89 (0.38, 2.05)	0.78	0.90 (0.36, 2.26)	0.83
Q4	0.93 (0.40, 2.16)	0.86	0.60 (0.21, 1.74)	0.35
		P trend = 0.97		*P* trend = 0.30
IEAA_Hannum				
Q1	1 (ref.)		1 (ref.)	
Q2	1.27 (0.52, 3.08)	0.60	0.31 (0.11, 0.82)	0.018
Q3	1.17 (0.47, 2.93)	0.74	0.37 (0.14, 0.96)	0.041
Q4	0.92 (0.40, 0.10)	0.84	0.35 (0.13, 0.92)	0.034
		P trend = 0.68		*P* trend = 0.03
IEAA_Horvath				
Q1	1 (ref.)		1 (ref.)	
Q2	0.85 (0.36, 2.00)	0.70	0.85 (0.34, 2.15)	0.73
Q3	0.73 (0.31, 1.75)	0.48	0.83 (0.33, 2.11)	0.70
Q4	0.21 (0.54, 2.74)	0.64	0.53 (0.17, 1.58)	0.25
		P trend = 0.65		*P* trend = 0.28

IEAA_Pheno				
Q1	1 (ref.)		1 (ref.)	
Q2	1.01 (0.43, 2.36)	0.98	1.34 (0.51,3.52)	0.55
Q3	1.10 (0.46, 2.63)	0.83	1.18 (0.46, 3.05)	0.73
Q4	1.04 (0.46, 2.35)	0.92	0.63 (0.21, 1.82)	0.39
		P trend = 0.88		*P* trend = 0.38

Abbreviations: IEAA: Intrinsic epigenetic age acceleration; ref.: referent. *Conditional logistic regression models, adjusting for batch effects, BMI, and smoking predicted pack years (using DNA methylation).

(*n* = 22 matched pairs; Supplementary Table 1). Finally, to examine whether associations might vary by histology, we separated NSCLC and SCLC; no associations were observed in either subgroup (data not shown).

Prior studies suggest that epigenetic age acceleration may be strongly linked to the immune response, and specifically CD8 and CD4 naïve cells [38]. In this study, all three epigenetic clock measures were strongly associated with CD8 and CD4 naïve immune subsets in control subjects (Pearson correlations ranging from −0.22 to −0.41; Supplementary Table 2). The only statistically significant correlation between the clock measures and NK cells was for PhenoAge (r = −0.16); however, the NK cells in the reference library do not differentiate naïve and memory NK cells, so it is possible the associations would be different for NK naïve cells. The CD8 memory cells were positively associated with Hannum and Horvath clocks but not with PhenoAge. The IEAA measures (for each clock) were not associated with CD8 memory cells but were still strongly associated with CD8 naïve cells, which can be explain by the lack of adjustment for naïve and memory T cells as these fractions were not available in earlier deconvolution libraries (and memory cell represent a larger proportion of total T cells in older adults).

DISCUSSION

In this nested case-control study on incident lung cancer, we observed no positive associations between lung cancer risk and epigenetic age acceleration using three different measures (Horvath, Hannum and PhenoAge) with two adjustment approaches for each, i.e., intrinsic and extrinsic measures. We observed inverse associations for men and subjects below the median age, but not in women or older subjects.

Our null findings for epigenetic age acceleration associations with lung cancer risk using the Horvath and Hannum clocks are consistent with those reported in a nested case-control study in the Melbourne Collaborative Cohort Study (MCCS; 332 cases) [18]. Our null findings differ from those reported in the Women's Health Initiative (WHI), where a 50% increase in risk of lung cancer was observed for every unit increase in intrinsic epigenetic age acceleration using the Horvath epigenetic age measure ($p = 3.4 \times 10^{-3}$) [17]; it is worth noting that the number of lung cancer cases included in the WHI analysis was small (*n* = 43). Our results for age acceleration based on the PhenoAge measure were also null, whereas a positive association was observed for PhenoAge and lung cancer risk in the MCCS (OR = 1.25, 95% CI = 1.05–1.49, for a 1 SD increase) [13] and in the WHI (HR = 1.05, *p* = 0.031) [16]. The PhenoAge measure was derived using immune and inflammatory phenotypes, in contrast to the other two epigenetic measures. Differences in the two populations may explain the different findings, such as smoking prevalence, although we could not confirm that as the MCCS analysis did not provide characteristics for the lung cancer case-control study (only for the pooled population). We observed that adjusting for methylation predicted pack-years attenuated the associations for PhenoAge in our analysis (among current smokers: before adjustment OR = 2.15, 95% CI = 0.92–5.04 for Q4 vs. Q1; after adjustment OR = 1.40, 95% CI = 0.52–3.76; overall: before adjustment OR 1.22, 95% CI = 0.70–2.13; after adjustment OR = 0.86, 95% CI = 0.46–1.62). Thus, it is possible that elevated risk associated with the PhenoAge age acceleration in some studies is a measure of the residual effect of smoking, which is captured with the methylation markers for pack-years smoked.

The inverse associations between age acceleration for several epigenetic clock measurements and lung cancer risk we observed in men and subjects less than 65 years of age were unexpected. However, our findings are consistent with results from a large study using Mendelian randomization (MR) methods conducted to examine the causal link between several epigenetic clocks and cancer, including lung cancer. In the MR analysis, the genetically predicted intrinsic Horvath Age acceleration was associated with a decrease in lung cancer risk (the association was statistically significant, *p* = 0.03, prior to multiple comparisons correction). Alternatively, these results may be due to a selection bias that occurred as a results of survivor bias in enrollment into our cohort (this could have occurrent if

individuals with poor health and epigenetic age acceleration were less likely to participate).

Recent studies have begun to elucidate the biological processes that explain age acceleration associations detected using epigenetic clocks [38]. In a functional genomics study, changes in proportions of naive and activated immune blood cells were strongly associated with the Hannum and Horvath age acceleration measures [38]. We confirmed these relationships in our dataset using new immune cell reference libraries allowing the deconvolution of naive immune T cells [22]. The strong inverse correlations observed between naive T and B cells and the three age acceleration measurements suggest that they are strongly linked to changes in the immune response, which is not surprising, given that reduction of naïve T cells is a component of immuno-senescence [39]. Of interest, the intrinsic epigenetic age acceleration (IEAA) measurements remained strongly associated with the CD8 naïve cells; future analyses using intrinsic measures of age acceleration should adjust for these cells.

The strengths of our study include the prospective nature of the analysis with a long follow-up period, thus removing the potential for spurious associations that may be driven by the cancer progression (i.e., reverse causation), a relatively large sample size for methylation analyses, and tight adjustment for smoking. In addition, the cancer ascertainment for the CLUE II cohort is very high, given the quality of cancer registry data. The limitations of our analysis include one-time point for epigenetic measurements and lack of data on non-Whites, thus limiting generalizability.

To our knowledge, this is the third prospective study examining the association between epigenetic aging, measured in peripheral blood, and risk of lung cancer. Findings from this study suggest that there are no strong positive associations between biological aging, measured an average of 15 years prior to cancer, and lung cancer risk. The majority of cases in this study were ever smokers (90%), and smoking history was well controlled for, suggesting that biological aging, independent of smoking, is not associated with an increased risk of lung cancer, at least among smokers. Our data also suggest that prior associations with PhenoAge and lung cancer might have been due to residual effects of smoking. Future studies should include more racially diverse populations and examine associations among never smokers.

AUTHOR CONTRIBUTIONS

DSM, KTK and EAP designed the study, obtained funding and acquired the data. JL and NZ assisted with preparation of dataset. DSM supervised all research activities. MC conducted the statistical analyses. DSM drafted the manuscript. MC, NZ, EAP, KTK, and DCK interpreted the data and provided critical revisions of the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

Dr. Kelsey is a founder and scientific advisor for Cellintec, which had no role in this research.

ETHICAL STATEMENT

The Institutional Review Board at the Johns Hopkins Bloomberg School of Public Health and the Tufts University Health Sciences Campus Institutional Review Board approved this study.

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SUPPLEMENTARY MATERIALS

Supplementary Tables

Supplementary Table 1. Odds ratios and 95% confidence intervals for the association between epigenetic age acceleration (using 3 different measures) and the risk of lung cancer in the CLUE II study, stratified by age and smoking status.

	OR (95% CI)*	*p*-value	OR (95% CI)*	*p*-value
	Age < 65 years		Age ≥ 65 years	
AA_Hannum	0.63 (0.44, 0.90)	0.011	0.90 (0.53, 1.52)	0.69
AA_Horvath	0.62 (0.44, 0.88)	0.007	1.10 (0.69, 1.75)	0.70
AA_Pheno	0.76 (0.58, 1.00)	0.046	1.07 (0.75, 1.52)	0.72
IEAA_Hannum	0.69 (0.48, 1.00)	0.05	0.91 (0.52, 1.59)	0.74
IEAA_Horvath	0.73 (0.52, 1.04)	0.08	1.08 (0.67, 1.74)	0.74
IEAA_Pheno	0.74 (0.55, 0.98)	0.035	1.13 (0.77, 1.67)	0.52
	Current smoker**		Former smoker**	
AA_Hannum	0.76 (0.49, 1.20)	0.24	0.69 (0.44, 1.07)	0.09
AA_Horvath	0.68 (0.45, 1.02)	0.06	0.82 (0.54, 1.27)	0.38
AA_Pheno	0.93 (0.68, 1.28)	0.66	0.84 (0.61, 1.16)	0.30
IEAA_Hannum	0.93 (0.58, 1.48)	0.76	0.71 (0.45, 1.12)	0.14
IEAA_Horvath	0.81 (0.53, 1.22)	0.31	0.91 (0.58, 1.43)	0.69
IEAA_Pheno	1.02 (0.73, 1.44)	0.90	0.85 (0.60, 1.20)	0.36

Abbreviation: IEAA: Intrinsic epigenetic age acceleration. *Conditional logistic regression models, adjusting for batch effects, BMI, and smoking predicted packyears (using DNA methylation). **Never smokers were not included in this analysis are there were too few.

Supplementary Table 2. Correlations between epigenetic clocks and selected immune cell proportions (estimated from methylation data).

	AA Hannum	IEAA Hannum	AA Horvath	IEAA Horvath	AA PhenoAge	IEAA PhenoAge
CD4 naïve	−0.3677	−0.1935	−0.1884	−0.0011	−0.2249	−0.1104
***p* values**	<0.0001	0.0051	0.0066	0.9877	0.0011	0.1124
CD8 naïve	−0.4245	−0.4076	−0.2305	−0.1706	−0.32	−0.3196
***p* values**	<0.0001	<0.0001	0.0008	0.014	<0.0001	<0.0001
CD8 memory	0.3316	0.1264	0.3202	−0.0026	−0.0203	0.098
***p* values**	<0.0001	0.0689	<0.0001	0.9706	0.7713	0.1591
B cell naïve	−0.2507	−0.1485	−0.1786	−0.0609	−0.3272	−0.1856
***p* values**	0.0003	0.0323	0.01	0.3831	<0.0001	0.0073

Correlations for CD4 memory and B cell memory are not shown as the correlations were statistically significant. Abbreviation: IEAA: Intrinsic epigenetic age acceleration.