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RESEARCH ARTICLE

Long-term ambient particle exposures and blood DNA methylation age: findings from the VA normative aging study

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Abstract

Background: Ambient particles have been shown to exacerbate measures of biological aging; yet, no studies have examined their relationships with DNA methylation age (DNAm-age), an epigenome-wide DNA methylation based predictor of chronological age. Objective: We examined the relationship of DNAm-age with fine particulate matter ($PM_{2.5}$), a measure of total inhalable particle mass, and black carbon (BC), a measure of particles from vehicular traffic. Methods: We used validated spatiotemporal models to generate 1-year $PM_{2.5}$ and BC exposure levels at the addresses of 589 older men participating in the VA Normative Aging Study with 1–3 visits between 2000 and 2011 ($n = 1032$ observations). Blood DNAm-age was calculated using 353 CpG sites from the Illumina HumanMethylation450 BeadChip. We estimated associations of $PM_{2.5}$ and BC with DNAm-age using linear mixed effects models adjusted for age, lifestyle/environmental factors, and aging-related diseases. Results: After adjusting for covariates, a 1-$\mu g/m^3$ increase in $PM_{2.5}$ (95% CI: 0.30, 0.75, $P < 0.0001$) was significantly associated with a 0.52-year increase in DNAm-age. Adjusted BC models showed similar patterns of association ($\beta = 3.02$, 95% CI: 0.48, 5.57, $P = 0.02$). Only $PM_{2.5}$ ($\beta = 0.54$, 95% CI: 0.24, 0.84, $P = 0.0004$) remained significantly associated with DNAm-age in two-particle models. Methylation levels from 20 of the 353 CpGs contributing to DNAm-age were significantly associated with $PM_{2.5}$ levels in our two-particle models. Several of these CpGs mapped to genes implicated in lung pathologies including *LZTFL1*, *PDLIM5*, and *ATPAF1*. Conclusion: Our results support an association of long-term ambient particle levels with DNAm-age and suggest that DNAm-age is a biomarker of particle-related physiological processes.

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Key words: Epigenetics; DNA methylation age; particulate matter 2.5; black carbon

Introduction

Annually, air pollution—including ambient particle exposures—contributes to 3.7 million deaths worldwide and is one of the world's largest single environmental health risks [1]. Emerging evidence has also suggested that ambient particles may have aging-related effects: particulate matter with aerodynamic diameter $\leq 2.5\,\mu m$ ($PM_{2.5}$) exposures have been associated with age-related outcomes including brain atrophy [2], declines in cognitive performance [3], ischemic heart disease [4], and stroke [5], as well as increases in systolic blood pressure by as much as 4.6 mmHg [6, 7]. Moreover, traffic related particle exposures have been associated with hastened lung function decline by 6–7% over a five year period [8], accelerated pigment spot formation, and other clinical hallmarks of premature skin aging [9]. Previous research has used telomere length (TL), a common biomarker of biological aging [10], to characterize the relationship between particle exposures and aging. Nevertheless, data on the associations between ambient particles and TL have been conflicting and thus reflect a need for alternative biological aging markers [6, 11, 12].

Recent developments in the epigenetics of aging have provided new opportunities to address the relationship between particle exposures and aging biology. DNA methylation is an epigenetic mark involved in regulating genomic structure and transcription [13]. Reproducible changes in DNA methylation have long been associated with chronological aging [14–16] and recent studies report persisting associations even after accounting for age-related cellular heterogeneity, a previously neglected confounder [17–19]. DNA methylation age (DNAm-age) is a novel tissue-independent predictor of chronological age and is calculated by an algorithm that uses methylation values from 353 chronological age-correlated CpG dinucleotides in Illumina's HumanMethylation450 BeadChip [20, 21]. Since DNA methylation in blood has been empirically shown to be sensitive to a number of biological processes [22–28], the DNAm-age of blood cells may help in further understanding epigenetic aging relationships with ambient particles. In this study, we investigated the relationship of DNAm-age with ambient particle exposures—$PM_{2.5}$ and black carbon (BC)—in a cohort of elderly men. We also examined the relationship of $PM_{2.5}$ and BC with leukocyte TL.

Results

Baseline Characteristics and Descriptive Statistics

All participants were Caucasian males with a mean age of 74.8 years (SD = 7.06) and a mean DNAm-age of 74.1 years (SD = 7.90, Table 1). Participants with coronary heart disease, hypertension, and a lifetime cancer diagnosis had a significantly higher mean DNAm-age than their respective counterparts (Supplementary Table S2). Furthermore, never smokers had a significantly higher mean DNAm-age when compared with former smokers (Supplementary Table S2). No significant associations were found when comparing never smokers to current smokers or current smokers to former smokers. Mean 1-year $PM_{2.5}$ and 1-year BC levels were 10.7 $\mu g/m^3$ (SD = 1.40) and 0.51 $\mu g/m^3$ (SD = 0.18), respectively (Table 1). Moreover, 1-year $PM_{2.5}$ and BC levels were significantly correlated ($r = 0.41$, $P < 0.0001$) in our study sample (Supplementary Table S3).

$PM_{2.5}$ and BC as Independent and Joint Predictors of DNAm-Age

Residuals from all models appeared normally distributed. In a model solely adjusted for chronological age and blood cell type, $1\,\mu g/m^3$ increases in 1-year $PM_{2.5}$ exposures were significantly associated with 0.55 year increases in DNAm-age ($P < 0.0001$). Following adjustments in Model 2, $PM_{2.5}$ remained associated with increases in DNAm-age ($\beta = 0.52$, $P < 0.0001$) (Table 2). These results remained consistent in Model 3 ($\beta = 0.52$, $P < 0.0001$) and Model 4 ($\beta = 0.50$, $P < 0.0001$), which adjusted for aging-related disease covariates and disease medications, respectively (Table 2). These $PM_{2.5}$ associations persisted in sensitivity

Table 1: descriptive statistics of study participants

Characteristic	
Number of observations (participants)	1032 (589)
Total number of visits, *n* (%)	
One	589 (57%)
Two	352 (34%)
Three	91 (9%)
Chronological age, mean (SD)	74.8 (7.06)
DNAm-age, mean (SD)	74.1 (7.90)
1-year $PM_{2.5}$ ($\mu g/m^3$), mean (SD)	10.7 (1.40)
1-year BC ($\mu g/m^3$), mean (SD)	0.51 (0.18)
Year average temperature (°C), mean (SD)	11.5 (1.19)
Cigarette pack years, mean (SD)	20.5 (24.4)
Relative TL, mean (SD)	1.25 (0.64)
Max years education, *n* (%)	
≤12 years	264 (25%)
12–16 years	493 (48%)
>16 years	275 (27%)
BMI, *n* (%)	
Underweight	2 (0%)
Healthy/Lean	234 (23%)
Overweight	549 (53%)
Obese	247 (24%)
Alcohol consumption, *n* (%)	
<2 drinks/day	831 (81%)
≥2 drinks/day	201 (19%)
Lifetime cancer diagnosis, *n* (%)	
Yes	574 (56%)
No	458 (44%)
Coronary heart disease, *n* (%)	
Yes	355 (33%)
No	677 (67%)
Diabetes, n (%)	
Yes	193 (19%)
No	839 (81%)
Hypertension, n (%)	
Yes	753 (73%)
No	279 (27%)
Smoking Status, n (%)	
Never	294 (29%)
Former	701 (67%)
Current	37 (4%)
Season, n (%)	
Spring	249 (24%)
Summer	245 (24%)
Fall	350 (34%)
Winter	188 (18%)

Table 2: 1-year $PM_{2.5}$ and BC as predictors of DNA methylation age (DNAm-age)

Particle (1 μg/m^3)	Difference in DNAm-age (95% CI)	*P*	*N*	AIC
$PM_{2.5}$				
Model 1	0.55 (0.33, 0.77)	**<0.0001**	1032	6346.85
Model 2	0.52 (0.30, 0.75)	**<0.0001**	1032	6360.86
Model 3	0.52 (0.29, 0.74)	**<0.0001**	1032	6361.47
Model 4	0.50 (0.27, 0.72)	**<0.0001**	1032	6362.88
BC				
Model 1	2.49 (0.11, 4.88)	**0.04**	898	5571.94
Model 2	3.02 (0.48, 5.57)	**0.02**	898	5583.16
Model 3	2.92 (0.36, 5.48)	**0.03**	898	5583.51
Model 4	2.83 (0.28, 5.39)	**0.03**	898	5582.92
Two-Particle Model 1			898	5560.38
$PM_{2.5}$	0.56 (0.28, 0.84)	**0.0001**		
BC	0.52 (−2.03, 3.08)	0.69		
Two-Particle Model 2			898	5574.56
$PM_{2.5}$	0.54 (0.24, 0.84)	**0.0004**		
BC	0.62 (−2.24, 3.47)	0.67		
Two-Particle Model 3			898	5575.71
$PM_{2.5}$	0.52 (0.22,0.83)	**0.0007**		
BC	0.61 (−2.25, 3.47)	0.67		
Two-Particle Model 4			898	5575.70
$PM_{2.5}$	0.51 (0.21,0.82)	**0.0009**		
BC	0.60 (−2.25, 3.46)	0.68		

Model 1: adjusted for chronological age and blood cell type. Model 2: Model 1 but additionally adjusted for temperature, pack years, smoking status, season, BMI, alcohol consumption, and education. Model 3: Model 2 but additionally adjusted for history of cancer, hypertension, chd, and diabetes. Model 4: Model 2 but additionally adjusted for statins and medications for diabetes and hypertension.

analyses with robust regression (data not shown) and in models adjusting for 450k plate, though the effect estimates were slightly attenuated (Supplementary Table S4). In a model adjusted for chronological age and blood cell type, BC was a significant predictor of DNAm-age ($\beta = 2.49$, $P = 0.04$), and remained a significant predictor of DNAm-age in subsequent models adjusting for additional covariates (Table 2). Nonetheless, after adjusting for 450k plate, the BC associations with DNAm-age remained marginally significant at best (Supplementary Tables S4). $PM_{2.5}$ levels remained significantly associated with increases in DNAm-age of 0.51 years or greater ($P < 0.0001$) in two-particle models with BC (Table 2) though the magnitude of the effect estimates were also attenuated following adjustments for 450k plate (Supplementary Table S4). BC levels were not significantly associated with DNAm-age in any of the two-particle models (Table 2 and Supplementary Table S4).

A sensitivity analysis exploring particle associations with DNAm-age in participants with only one Normative Aging Study (NAS) visit, revealed similar, but non-significant trends as the primary analysis (Supplementary Table S5). A subsequent sensitivity analysis that stratified the study sample by season of NAS visit also revealed similar trends as the primary analysis, but results were only significant for $PM_{2.5}$ associations in the summer and fall NAS visit groups (Supplementary Table S6). Finally, an analysis using participants with at least two NAS visits and exploring the correlation between the change in particle exposure between visits and the change in DNAm-age between visits, revealed weak and non-significant correlations (Supplementary Table S7).

Associations between $PM_{2.5}$ Levels and Methylation Values at Individual DNAm-Age CpG Sites

We explored associations between $PM_{2.5}$ levels and the methylation values for the 353 CpG sites that are used to calculate DNAm-age. Methylation of 20 out of 353 CpGs was significantly associated with $PM_{2.5}$ levels in two-particle mixed effects Model 2 (adjusting for BC, age, blood cell type, and lifestyle/environmental characteristics) following FDR correction (Fig. 1). $PM_{2.5}$ levels were positively or negatively associated with CpG methylation depending on the CpG site (Table 3). The 20 CpGs mapped to 20 known genes; nevertheless, gene ontology analysis did not return significant pathway enrichment (data not shown). No CpGs were significantly associated with BC levels in the two-particle mixed effects model.

DNAm-Age, $PM_{2.5}$, and BC as Predictors of Relative TL

TL showed a weak and non-significant correlation ($r = -0.06$, $P = 0.08$) with DNAm-age in participants' NAS observations (Supplementary Table S3). Moreover, DNAm-age was not a significant predictor of TL in mixed effects models adjusting for chronological age, blood cell type, and telomere batch (Table 4). TL also showed no significant associations with 1-year $PM_{2.5}$ or 1-year BC levels in any of the single-particle or two-particle models (Table 5).

Discussion

This study showed a novel positive association between 1-year $PM_{2.5}$ exposure levels and DNAm-age. To the best of our knowledge, this is the first study showing relationships between any environmental pollutant and an epigenetic biomarker of aging. $PM_{2.5}$ remained a statistically significant positive predictor of DNAm-age after adjusting for chronological age and other covariates. The study also revealed a significant positive association between BC and DNAm-age after adjusting for age and other covariates, but not after adjusting for 450k plate. Moreover, we identified 20 age-related CpG sites whose methylation was

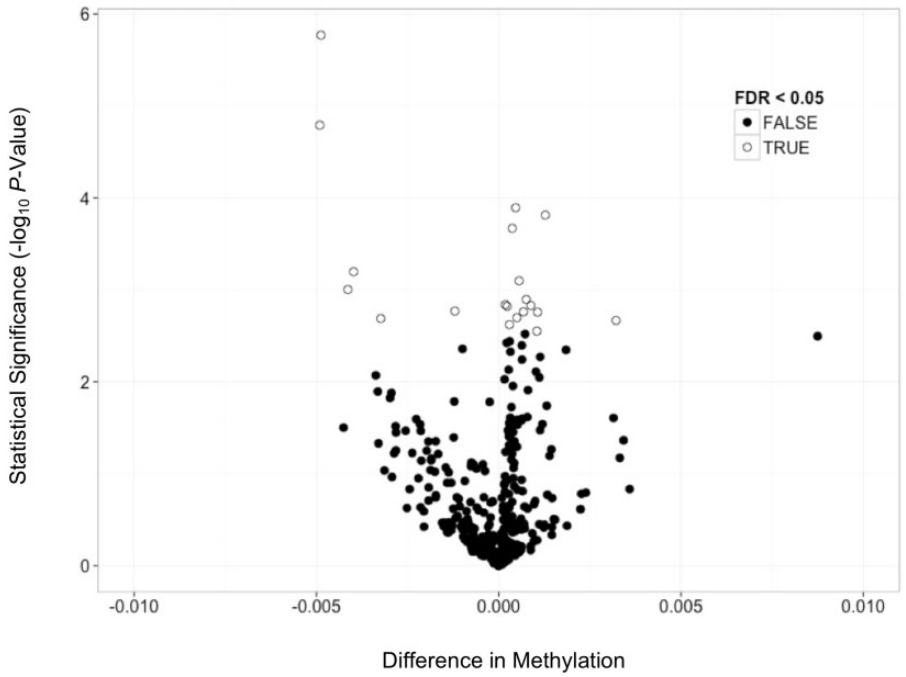

Figure 1: volcano plot of regression coefficients for difference in DNA methylation beta values from 353 DNAm-age CpGs analyzed for association with 1-year $PM_{2.5}$ levels in a two-particle model. Linear mixed effects models were used to explore the associations between 1-Year $PM_{2.5}$ exposure levels and DNA methylation values for the 353 CpG sites used to calculate DNAm-age. The regression coefficient for the difference in DNA methylation beta values given by a 1 $\mu g/m^3$ increase in 1-year $PM_{2.5}$ exposure level is plotted on the x-axis, and the corresponding significance is plotted on the y-axis. CpG probes meeting statistical significance following FDR adjustment are depicted as hollow circles. DNA methylation beta values range from 0 (completely unmethylated) to 1 (completely methylated).

Table 3: 1-year $PM_{2.5}$ as a predictor of CpG probe methylation in a two-particle model

CpG[a]	Gene	Process	Difference in methylation	*P*	FDR
Negative association					
cg14163776	ACAP2	GTPase activator activity	−0.0049	**<0.0001**	**0.003**
cg06044899	TMSL3	actin cytoskeleton organization	−0.0048	**<0.0001**	**0.001**
cg01570885	FAM50B	protein binding	−0.0041	**0.001**	**0.041**
cg18139769	SGCE (PEG10)	calcium ion binding	−0.0040	**0.001**	**0.037**
cg22736354	NHLRC1	ubiquitin-protein transferase activity	−0.0032	**0.002**	**0.042**
cg15661409	C14orf105	uncharacterized	−0.0012	**0.002**	**0.041**
Positive association					
cg02047577	UCKL1	uridine kinase activity	0.0002	**0.001**	**0.041**
cg10940099	CD164	cellular adhesion	0.0002	**0.002**	**0.041**
cg22006386	CATSPERG	ion channel activity	0.0003	**0.002**	**0.044**
cg08186124	LZTFL1	protein binding: cytoplasm	0.0004	**<0.0001**	**0.015**
cg04094160	ZBTB5	transcriptional regulation	0.0005	**<0.0001**	**0.014**
cg16408394	RXRA	DNA binding	0.0005	**0.002**	**0.042**
cg23786576	ATPAF1	ATP synthase complex assembly	0.0006	**0.001**	**0.040**
cg15341340	DNASE2	endodeoxyribonuclease activity	0.0007	**0.002**	**0.041**
cg21395782	NDUFA13	NADH dehydrogenase activity	0.0008	**0.001**	**0.041**
cg26043391	FBXO28	protein binding	0.0009	**0.001**	**0.041**
cg06557358	TMEM132E	integral component of membrane	0.0010	**0.003**	**0.050**
cg14409958	ENPP2	nucleic acid binding	0.0011	**0.002**	**0.041**
cg20305610	PDLIM5	actin binding	0.0013	**<0.0001**	**0.014**
cg05675373	KCNC4	potassium channel activity	0.0032	**0.002**	**0.042**

[a]adjusted for chronological age, blood cell type, BC, temperature, pack years, smoking status, season, BMI, alcohol consumption, education, and 450k technical covariates.

significantly associated with $PM_{2.5}$ exposure levels in two-particle models adjusting for BC, age, and other covariates.

Operating under the premise that adverse exposures accelerate aging, we expected 1-year $PM_{2.5}$ and BC exposure levels to be associated with increases in DNAm-age. In alignment with our expectations, both $PM_{2.5}$ and BC exposure levels were positively associated with DNAm-age. Pearson correlations of between visit changes in particle exposures and between visit

Table 4: DNAm-age as a predictor of relative TL

	Change in TL (95% CI)	*P*	*n*	AIC
DNAm-age				
Model 1	−0.006 (−0.01, 0.002)	0.14	857	1687.65
Model 2	−0.004 (−0.01, 0.002)	0.23	856	1233.57

Model 1: adjusted for chronological age, blood cell type, and telomere batch. Model 2: Model 1 but excluding one participant with an outlying telomere value.

Table 5: 1-year $PM_{2.5}$ and BC as predictors of TL

Particle (1 μg/m^3)	Difference in TL (95% CI)	*P*	*n*	AIC
$PM_{2.5}$				
Model 1	0.02 (−0.01, 0.06)	0.24	857	1750.26
Model 2	0.02 (−0.01, 0.06)	0.20	857	1754.13
Model 3	0.02 (−0.01, 0.06)	0.23	857	1776.27
Model 4	0.02 (−0.01, 0.06)	0.21	857	1771.64
BC				
Model 1	0.11 (−0.16, 0.38)	0.42	770	1616.52
Model 2	0.13 (−0.16, 0.42)	0.37	770	1637.87
Model 3	0.12 (−0.16, 0.41)	0.40	770	1658.35
Model 4	0.13 (−0.16, 0.42)	0.38	770	1654.71
Two-particle Model 1			770	1623.11
$PM_{2.5}$	0.02 (−0.02, 0.07)	0.32		
BC	0.05 (−0.24, 0.35)	0.71		
Two-particle Model 2			770	1644.69
$PM_{2.5}$	0.02 (−0.03, 0.07)	0.40		
BC	0.07 (−0.25, 0.38)	0.66		
Two-particle Model 3			770	1665.33
$PM_{2.5}$	0.02 (−0.03, 0.07)	0.47		
BC	0.07 (−0.24, 0.39)	0.65		
Two-particle Model 4			770	1661.52
$PM_{2.5}$	0.02 (−0.03, 0.07)	0.39		
BC	0.07 (−0.25, 0.39)	0.67		

Model 1: adjusted for chronological age and blood cell type. Model 2: Model 1 but additionally adjusted for temperature, pack years, smoking status, season, telomere batch, BMI, alcohol consumption, and education. Model 3: Model 2 but additionally adjusted for history of cancer, hypertension, chd, and diabetes. Model 4: Model 2 but additionally adjusted for statins and medications for diabetes and hypertension.

changes in DNAm-age in participants with multiple visits were not significant potentially due to the smaller number of observations. Nonetheless, compared with the primary analysis, we observed similar trends in the association of our particles with DNAm-age in sensitivity analyses using participants with a single NAS visit. These trends suggest that having a single or multiple visits was not driving the results from the adjusted mixed effects models. Likewise, trends similar to the primary analysis were also observed in our seasonal analysis and were significant for the summer and fall seasons, which had the highest average particle exposures across all observations.

Although DNAm-age is primarily viewed as a predictor of chronological age, emerging research suggests that it reflects underlying physiological processes including metabolic dysregulation, immune dysfunction, and genomic instability [29–32]. To date, two studies have described significant associations between DNAm-age and all-cause mortality [29, 33]. Moreover, studies have also demonstrated that DNAm-age may predict or be reflective of various disease processes [31, 32, 34–37]. It is hypothesized that DNAm-age may measure 'the cumulative work done by a particular kind of epigenetic maintenance system (EMS), which helps maintain epigenetic stability' [20]. Under the EMS hypothesis, an increase in DNAm-age suggests that an event or process has occurred and the EMS has completed more work to repair or return the epigenome to homeostasis. Alternatively, a reduction in DNAm-age can be interpreted as epigenetic stability or disrupted activation of the EMS, both of which would result in less maintenance work. Given the known toxicity of ambient particles, our data supports the theory that some particles may disrupt the epigenome thus requiring more maintenance work. Nevertheless, mechanistic studies are warranted to explicitly identify the components of this system.

Given our interpretation of the relationship between adverse exposures and DNAm-age, it was interesting to find that cigarette pack years was negatively correlated with DNAm-age and that former smokers had a lower mean DNAm-age than never smokers. Though cigarette smoking can be considered a personal form of air pollution, it is also a complex mixture with a composition that differs from that of $PM_{2.5}$. Differences in particle composition can account for differences in the toxicological pathways of these exposures and may be one reason why we observe differences in their DNAm-age relationships. Moreover, individuals who are sick are often urged to quit smoking so there may still be some confounding when observing the unadjusted correlations of pack years and cigarette smoking status with DNAm-age. A number of physiological factors can also affect the epigenome and should be considered when comparing smoking to air pollution exposures. For instance, it is widely known that smoking can account for substantial weight loss and it has been demonstrated that obesity accelerates the DNAm-age of liver cells [31]. Finally, a study sample with 37 current smoker observations may be underpowered to detect differences in mean DNAm-age between current smokers and other groups.

In our two-particle models, BC exposure levels were not significantly associated with DNAm-age while $PM_{2.5}$ remained a significant predictor of DNAm-age. BC is considered a specific marker of traffic-related air pollution, while $PM_{2.5}$ is a heterogeneous mixture of fine particles with component species often including carbonaceous fractions (e.g. BC), inorganic compounds (e.g. sulfate, nitrate, ammonium), and trace metals (e.g. nickel, lead, copper) [38]. Research on total $PM_{2.5}$ is more extensive than any work singularly exploring BC or other components. Many studies suggest that BC may be more toxic than $PM_{2.5}$ [39], but data also exists where $PM_{2.5}$ associations are stronger than that of BC [40]. The finding that $PM_{2.5}$ was driving the association with DNAm-age in the two particle models may possibly be because other components apart from BC are responsible for the DNAm-age relationship. Another theory is that the mixture of the $PM_{2.5}$ components is more harmful, in regards to DNAm-age, than any of the components singularly. Ultimately, further work involving a detailed compositional analysis of $PM_{2.5}$ will aid in further understanding what components are driving the associations with DNAm-age.

Although we attributed the observed positive association of $PM_{2.5}$ with DNAm-age to greater cumulative work by the EMS, we also conducted additional analyses to identify which of the 353 CpG sites contributing to the DNAm-age metric had methylation values that were significantly associated with $PM_{2.5}$ levels. We identified 20 such CpGs through a mixed effects model adjusting for chronological age, blood cell type, and lifestyle/environmental factors. These CpGs mapped to 20 known genes. A gene ontology analysis of these 20 genes did not return any significant enrichment for specific biological pathways. Nevertheless, a literature review revealed relationships

between the genes. For instance, *LZTFL1*, *PDLIM5*, and *ATPAF1*, can all be generally characterized as being involved in protein binding. *LZTFL1* (Leucine Zipper Transcription Factor-like 1) is a nuclear gene that encodes a cytoplasmic protein that interacts with other cytosolic proteins to regulate ciliary trafficking and control β-catenin nuclear signaling. *LZTFL1* downregulation has been implicated in non-small cell lung cancer and poor survival. In contrast, *LZTFL1* re-expression in lung tumor cells inhibits tumor growth and lung tissue colonization by circulating tumor cells [41]. *ATPAF1* (ATP Synthase Mitochondrial F1 Complex Assembly Factor 1) encodes a soluble mitochondrial protein that helps prevent abnormal aggregation of F1-ATP synthase subunits, and, like *LZTFL1*, is expressed in many tissues including the lung. *ATPAF1* is highly expressed in bronchial biopsies of individuals with severe asthma and has been found to predispose children of different ancestries to asthma [42]. Unlike *LZTFL1* and *ATPAF1*, *PDLIM5* (PDZ and LIM domain 5) primarily is involved in cardiomyocte function. Nonetheless, *PDLIM5* still has implications for lung physiology as its downregulation has been linked to hypoxia-induced pulmonary hypertension [43]. Collectively, our data suggests putative relationships between ambient particle levels and genes involved in various elements of lung physiology. Nonetheless, additional methylation and mechanistic studies will be necessary to first confirm these changes in gene methylation and next ascertain if these changes actually manifest themselves as differences in gene expression and protein levels/activity.

Finally, to help interpret our DNAm-age results, we explored the relationship of $PM_{2.5}$ and BC exposure levels with relative TL. Telomeres are nucleoprotein structures, at the ends of eukaryotic chromosomes, involved in maintaining genomic fidelity. Telomere shortening has been associated with aging and aging-related diseases [44]. Contrary to our expectations, we observed no association of $PM_{2.5}$ and BC with relative TL. As mentioned, the literature examining the relationship between particles and TL has been conflicting. Significant associations between annual $PM_{2.5}$ exposures and decreased TL have been reported [45], but in the NAS the relationship between annual BC exposures and decreased TL was only observed in never smokers [11]. The literature concerning short-term particle exposures is even more obscure. In some cases, short-term particle exposures have been associated with increased TL [46], decreased TL [47], and in other instances no significant association was observed [6, 48]. Our findings add to the body of literature that suggests: (i) that exposure duration and study population characteristics are particularly critical in understanding and interpreting the results of TL studies; and (ii) other measures, like DNAm-age, may offer more advantages for understanding the relationship between particle exposures and biological aging. Moreover, DNAm-age was not associated or correlated with TL in our study sample. Similar non-significant relationships between DNAm-age and TL have also been independently reported in a study conducted in the Lothian Birth Cohorts [49]. The known relationships of DNAm-age and TL with *in vitro* cell passaging also highlight the differences between these markers. As cells are passaged, they divide and in most cases their telomeres shorten [50]. However, DNAm-age increases as cells are passaged and divide *in vitro* [20]. In all, our findings and existing evidence suggests that though DNAm-age and TL are both measures of 'aging', the two are not one in the same and may capture different elements of biological processes.

Though we present a study with a number of objective, validated measures and rigorous statistical methods, our study does have a few limitations. First, our $PM_{2.5}$ and BC measurements were generated using spatiotemporal prediction models. Though the models were validated [51], we cannot completely eliminate residual measurement error or discrepancies in calibration coefficients [52]. Also, ambient levels of air pollution at a participant's address may differ from personal exposures, which also depend on time spent at home, rates of penetration of ambient particles into the house, and the presence of indoor sources of particles. However, we note that the demographics of the Normative Aging Study, which is composed primarily of retired older men, make it more likely that participants spend a large part of their day at home. Our findings are also based on a cohort of older Caucasian males residing in a lightly polluted urban environment; thus, studies including younger individuals, females, non-Caucasians, and in different environments are warranted to confirm our findings more broadly. Last, we attempted to adjust for potential confounders, but cannot rule out the possibility of unknown or residual confounding in our analyses.

Conclusion

Our data suggests that DNAm-age and TL capture different elements of biological aging; describes novel associations between ambient particles and DNAm-age; and highlights existing limits in interpretations of biological/molecular aging. Further analyses utilizing DNAm-age with $PM_{2.5}$, BC, and other particles may provide much needed insight into fully understanding the biologically adverse nature of ambient particles.

Materials and methods

Study Population

The NAS is an ongoing longitudinal cohort study of male volunteers within the Eastern Massachusetts community established in 1963 by the US Department of Veterans Affairs (VA). Participants free of any chronic medical conditions were enrolled in the study and returned for onsite, detailed medical examinations every 3–5 years, during which data on stress levels, diet, physical activity, smoking status, and additional risk factors that may impact health were collected. Participants provided written informed consent to the VA Institutional Review Board (IRB). The Harvard T.H. Chan School of Public Health and the VA IRBs granted human subjects approval.

Eligibility for our study sample required continued participation as of 2000, when $PM_{2.5}$ air pollution levels became available. We excluded NAS participants with a diagnosis of leukemia (11 participants) because of a possible influence on the DNA methylation of blood cells. The remaining 589 participants were used in the analysis (Supplementary Fig. S1). Study staff measured DNA methylation on blood DNA collected at up to three different visits for the participants. Using all available visits for each participant resulted in 1032 total observations.

Assessment of Environmental Factors: Ambient Particles and Temperature

We selected $PM_{2.5}$ and BC as our ambient particle exposures because of their global pervasiveness [1] in addition to their status as the leading ambient particles with well-documented relationships with both DNA methylation [53–55] and adverse health outcomes [56–59].

To generate daily $PM_{2.5}$ exposure levels (in $\mu g/m^3$) at each participant's address, we employed a well-validated satellite based hybrid spatiotemporal prediction model with a multi-step approach [60, 61]. The hybrid model combined satellite-derived aerosol optical depth (AOD) measurements and local land use regression model variables (e.g. traffic density, population density, and elevation) alongside temporal variables (e.g. temperature, wind speed, etc.). We fit the models to data from each year separately and generated daily predictions at the 1×1 km area resolution. Each participant's residence was geocoded and linked to an area level grid-point. To create a metric of long-term exposure, we averaged daily $PM_{2.5}$ level predictions at each participant's address over the 365 days prior to the day of visit. The prediction model had an out of sample R^2 of 0.88 for daily samples.

We generated daily BC exposure estimates (in $\mu g/m^3$) based on participants' residences using a validated spatiotemporal land-use regression model [62]. Daily average BC estimates from 83 monitoring sites throughout the Greater Boston area were used to develop a prediction model. The final model included predictors based on information from meteorological conditions (e.g. wind speed), land use (e.g. traffic density), daily BC concentrations at a central monitor, and additional descriptors (e.g. day of the week). The prediction model had a high R^2 of 0.83 based on the training data set and a moderate correlation between predicted values and observed BC levels in four out-of-sample validation samples ($R^2 = 0.59$). To generate a 1-year BC exposure, we averaged daily BC exposure levels for the 365 days prior to the day of NAS visit.

To generate ambient temperature (in Celsius) for each participant we used a spatiotemporal prediction multi-step approach [51]. We obtained daily physical surface temperature (T_s) data from AOD measurements with 1×1 km resolution and daily near surface air temperature (T_a) data from the National Climatic Data Center, Environmental Protection Agency, and Weather Underground Inc. Mixed model regression was first used to calibrate T_s to T_a in 1×1 km grid cells where both were available. The model was validated with mean out of sample R^2 for days with available T_s and days without T_s equal to 0.95 and 0.94, respectively. Daily temperature measurements were averaged over the 365 days prior to the visit to generate 1-year temperature exposure estimates to complement the 1-year $PM_{2.5}$ and BC measurements. We selected the 1-year average because it correlates well with averages of $PM_{2.5}$, BC, and temperature over longer time windows and was available for a higher number of participants (Supplementary Table S1). Moreover, existing studies examining relationships between particle exposures and other biological markers of aging, like TL, report more consistent and biologically significant results when a 1-year particle exposure is utilized [6, 11, 45–48].

DNA Methylation and Calculation of DNAm-Age

Laboratory staff extracted DNA from buffy coat of 7 ml whole blood using the QIAamp DNA Blood Kit (QIAGEN, Valencia, CA, USA). 500 ng DNA samples were then treated for bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research, Orange, CA, USA). Following bisulfite conversion, DNA samples were hybridized to the 12 sample Illumina HumanMethylation450 BeadChips as per Infinium HD Methylation protocol (Illumina, San Diego, CA, USA). Study staff then used a two-stage age-stratified algorithm to randomize samples to avoid confounding with chip and plate effects while ensuring similar age distribution across chips and plates. For quality control, we removed samples where >5% of probes had beadcount < 3 and > 1% of probes had a detection *P*-value >0.05. The Bioconductor minfi package Illumina-type background correction without normalization was used to preprocess the remaining samples and generate methylation beta values to compute DNAm-age [63]. 450k arrays were run in the Genomics Core Facility at Northwestern University.

We calculated DNAm-age through Horvath's publically available online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/) [20]. In short, an elastic net model (penalized regression) was used to regress a calibrated version of chronological age on 21 369 CpG probes shared by Illumina HumanMethylation27 and HumanMethylation450 BeadChip platforms. The elastic net platform selected 353 CpGs that correlate with age (193 positively and 160 negatively). The calculator predicts the age of each DNA sample (DNAm-age) using regression coefficients of the 353 CpGs resulting from the elastic net regression model trained from a number of training data sets. The calculator maintains predictive accuracy (age correlation 0.97, error = 3.6 years) across body tissues including blood [20].

Assessment of Leukocyte TL

Laboratory staff performed quantitative real time polymerase chain reaction (qRT-PCR) on DNA extracted from buffy coat of whole blood using the QIAamp DNA Blood Kits [64]. Relative TL was measured on a 7900HT Fast RT-PCR System (Applied Biosystems, Foster City, CA, USA) as the qRT-PCR factor by which a sample differs from a reference DNA sample in its ratio of telomere repeat copy number (T) to single *36B4* gene copy number (S) [11, 64]. The *36B4* gene is located on chromosome 12 and encodes acidic ribosomal phosphoprotein PO. Laboratory staff ran all samples in triplicate and derived the average T:S ratio by dividing the average of the three T measurements by the average of the three S measurements. TL was then reported in relative units (qRT-PCR factor) of T:S ratio in the test sample to T:S ratio in the reference DNA pool. Batches for participant qRT-PCR telomere measurements were also recorded.

Assessment of Smoking Status

Smoking histories were collected on all study participants at NAS entry and standardized smoking interviews were administered at each subsequent NAS visit. Smoking status was characterized into three groups: (i) 'never smokers' were individuals who reported at entry and consistently thereafter that their lifetime cigarette consumption was < 100 cigarettes; (ii) 'former smokers' reported that they had smoked in the past but quit prior to study entry or they were smokers at entry and quit at some point during the follow up period and remained quit at the present study visit; (iii) 'current smokers' were those who reported smoking regularly at each the follow up visit or those who quit, but reported inability to maintain abstinence at the present study visit. All participants also reported their average number of cigarettes per day at each assessment.

Statistical Analysis

We used generalized linear mixed effects models to evaluate the relationship of DNAm-age with 1-year $PM_{2.5}$ and 1-year BC exposure levels, singularly and in two-particle models. To account for within participant correlation between the repeated measurements, the mixed effects models included a random intercept for each participant. DNAm-age, 1-year $PM_{2.5}$, and 1-year BC were all considered as continuous variables in all analyses.

The aforementioned models were adjusted for known confounders and covariates with *a priori* biological/clinical relevance using a tiered approach. Given that results from previous DNA methylation studies have been confounded by blood cell heterogeneity, we obtained cell type estimates for six blood cell types (i.e. plasma, CD4T, CD8T, NK, monocytes and granulocytes) using Houseman and Horvath methods [20, 65]. We first constructed chronological age and blood cell type adjusted mixed effects models for the relationships of $PM_{2.5}$ and BC with DNAm-age (Model 1). Next, we built models (Model 2) accounting for environmental/lifestyle factors by adjusting for average 1-year temperature (continuous), cumulative cigarette pack years (continuous), smoking status (current, former, or never), and season of visit (Spring [March–May], Summer [June–August], Fall [September–November], and Winter [December–February]), body mass index (BMI) (lean [<25], overweight [25–30], obese [>30]), alcohol intake (yes/no $\geq$ 2 drinks daily), and maximum years of education (continuous) in addition to the Model 1 covariates. We constructed a third (Model 3) and fourth set of models (Model 4) which accounted for aging-related diseases and disease-related medications respectively. Model 3 adjusted for cancer (yes/no history of lifetime cancer diagnosis), coronary heart disease (yes/no based on electrocardiogram, validated medical records, or physical exam), diabetes (physician diagnosis or a fasting blood glucose > 126 mg/dl), and hypertension (yes/no antihypertensive medication use or systolic blood pressure $\geq$140 mmHg or diastolic blood pressure $\geq$90 mmHg) in addition to the Model 2 covariates. Model 4 adjusted for subjects taking statins and/or any diabetes and hypertension medications in addition to the Model 2 covariates. Last, we constructed two-particle mixed effects models with both $PM_{2.5}$ and BC as predictors of DNAm-age using the covariates from the Model 1–4 framework.

To exclude sensitivity of our models to outliers, we repeated all analyses using robust regression. By iteratively reweighting data points such that points far from model predictions in the previous iteration are given smaller weights, robust regression is able to minimize the sensitivity of a model to outlying values. Iterations continue until the values of coefficient estimates meet a specified tolerance and weighted least squares regression is then used to compute model coefficients. We performed a set of additional sensitivity analyses: (i) we added a random intercept for 450k plate to account for potential batch effects, (ii) we explored our particle DNAm-age associations in participants with only one NAS visit to see how our results compared with the primary analysis on the full study sample and (iii) we stratified our study sample by season of NAS visit to further explore the contribution of season to the relationship between particle exposures and DNAm-age. We also looked at the Pearson correlation between change in particle exposure and change in DNAm-age between study visits using participants with at least two NAS visits.

Additionally, we evaluated the relationships of DNA methylation values at each of the 353 DNAm-age CpG probes with 1-year $PM_{2.5}$ and 1-year BC exposure levels using the aforementioned Model 2 covariates and technical covariates (450k plate, chip, row, and column). FDR correction was performed to account for multiple hypotheses testing for all CpG methylation analyses. Gene ontology analyses were performed on significant CpG results using the publically available DAVID bioinformatics platform [66, 67].

As a means of comparison with the DNAm-age results, we explored the relationships of a standard marker of aging, TL, with $PM_{2.5}$ and BC exposure levels. We constructed mixed effects multivariable linear regression models adjusting for chronological age, blood cell type, average 1-year temperature, cumulative cigarette pack years, smoking status, season of visit, telomere batch (categorical with four batches), BMI, alcohol intake, and maximum years of education. Similar to our DNAm-age analyses, we constructed two additional sets of models adjusting for age-related diseases and disease-related medications respectively. There was one relative TL observation of 12.7, while the remaining 856 TL observations were < 4. We kept the outlying observation in the TL mixed effects models, but re-ran the models using robust regression and without the outlying value as sensitivity analyses.

We performed all statistical analyses using R Version 3.1.1 (R Core Team, Vienna, Austria) and considered a P-value < 0.05 to be statistically significant.

Contributors

J.N.E., E.C., and A.A.B. conceived and designed the study. L.T., I.K., A.C.J., Ji.S., K.B., A.D., L.H., and P.V. gathered data. JNE performed the data analyses and drafted the article. J.S., E.C., L.T., Ji.S., and A.C.J. contributed to the analyses. All authors revised and approved the article.

Ethics approval

Boston VA Medical Center, Harvard T.H. Chan School of Public Health (protocol 14027-102).

Conflict of interest statement. None declared.

Data Availability

Data are from the Normative Aging Study, from which restricted data are available for researchers who meet the criteria. A subset of the methylation data is deposited at NCBI dbGaP (study accession number: phs000853.v1.p1).

Supplementary data

Supplementary data is available at EnvEpig online.

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