

Associations between Long-term Exposure to PM_{2.5} Component Species and Blood DNA Methylation Age in the Elderly: The VA Normative Aging Study

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Abstract

Background: Long-term PM_{2.5} exposure and aging have been implicated in multiple shared diseases; studying their relationship is a promising strategy to further understand the adverse impact of PM_{2.5} on human health.

Objective: We assessed the relationship of major PM_{2.5} component species (ammonium, elemental carbon, organic carbon, nitrate, and sulfate) with Horvath and Hannum DNA methylation (DNAm) age, two DNA methylation-based predictors of chronological age.

Methods: This analysis included 552 participants from the Normative Aging Study with multiple visits between 2000 and 2011 (n=940 visits). We estimated 1-year PM_{2.5} species levels at participants' addresses using the GEOS-chem transport model. Blood DNAm-age was calculated using CpG sites on the Illumina HumanMethylation450 BeadChip. We fit linear mixed-effects models, controlling for PM_{2.5} mass and lifestyle/environmental factors as fixed effects, with the adaptive LASSO penalty to identify PM_{2.5} species associated with DNAm-age.

Results: Sulfate and ammonium were selected by the LASSO in the Horvath DNAm-age models. In a fully-adjusted multiple-species model, interquartile range increases in both 1-year sulfate (95%CI: 0.28, 0.74, $P<0.0001$) and ammonium (95%CI: 0.02, 0.70, $P=0.04$) levels were associated with at least a 0.36-year increase in Horvath DNAm-age. No PM_{2.5} species were selected by the LASSO in the Hannum DNAm-age models. Our findings persisted in sensitivity analyses including only visits with 1-year PM_{2.5} levels within US EPA national ambient air quality standards.

Conclusion: Our results demonstrate that sulfate and ammonium were most associated with Horvath DNAm-age and suggest that DNAm-age measures differ in their sensitivity to ambient particle exposures and potentially disease.

Keywords: DNA methylation age; Particulate matter 2.5; Long-term exposure; Epigenetics

1 | Introduction

Fine particulate matter (PM_{2.5}) remains an inescapable environmental exposure and an enormous global public health concern [1]. It is estimated that at least 2.1 million lives could be saved annually if PM_{2.5} guidelines were adhered to worldwide [2]. For the millions of people exposed to PM_{2.5} daily, understanding the impact of PM_{2.5} on human health is critical for developing interventions aimed at reducing PM_{2.5}-related morbidity and mortality globally. Researchers have consistently demonstrated that long-term PM_{2.5} exposure is a major contributor to cardiopulmonary disease [3-8], and emerging evidence suggests that PM_{2.5} is a risk factor for previously unconsidered disease outcomes like cognitive decline [9-11]. Nevertheless, much remains to be understood about how PM_{2.5} contributes to even its most well-documented disease outcomes. One promising strategy to better understand the adverse impact of PM_{2.5} on human health, is to study the relationship of PM_{2.5} with aging. Many studies have implicated PM_{2.5} as a contributor to accelerated aging [12-17]. Moreover, independent of PM_{2.5} exposures, aging is associated with cardiopulmonary disease, cognitive decline, and many other PM_{2.5}-related disease outcomes [18-21]. Thus, understanding how PM_{2.5} can contribute to aging, may provide additional insight into other adverse PM_{2.5}-related health effects.

DNA methylation-based biomarkers of age have proved to be promising tools in understanding the relationship of PM_{2.5} with aging. These biomarkers have surpassed their initial utility of simply predicting chronological age, and have demonstrated remarkable usefulness in assessing individuals' risk of mortality, malignancy, neurocognitive disease, and other biologically-relevant health endpoints [22-28]. Evidence also suggests that these biomarkers of age are

reflective of individuals' past environmental exposures [29]. One such study by our group demonstrated robust associations between $PM_{2.5}$ exposure levels and Horvath DNA methylation (DNAm) age. Horvath DNAm-age is a tissue-independent predictor of chronological age that is calculated from DNA methylation values at 353 chronological age-correlated CpG dinucleotides in Illumina's HumanMethylation450 BeadChip [30]. Specifically, in an elderly cohort and with fully-adjusted models, we observed that a $1 \mu\text{g}/\text{m}^3$ increase in 1-year $PM_{2.5}$ exposure was associated with a 0.52-year increase in Horvath DNAm-age [31].

Still, $PM_{2.5}$ is a heterogeneous mixture of carbonaceous fractions, inorganics, and metals; and it is widely appreciated that $PM_{2.5}$ component species often differ in their health effects [32-35]. The present study builds upon our previous research and examines the relationships of $PM_{2.5}$ component species with both Horvath and Hannum DNAm-age in elderly men. Hannum DNAm-age is also a DNA methylation-based predictor of chronological age, but it is based on measurements from 71 CpG dinucleotides [36]. Only 6 CpG dinucleotides are shared between the Horvath and Hannum metrics. By investigating the relationships of $PM_{2.5}$ component species with these two forms of DNAm-age, we aim to (1) better understand how specific $PM_{2.5}$ species are related to aging, and (2) demonstrate differences in the biological utility of different DNAm-age measures.

2 | Materials and Methods

2.1 Study population

The participants in this analysis were part of the U.S. Veterans Affairs Normative Aging Study (NAS), a longitudinal investigation of aging men established in Eastern Massachusetts in 1963

[37]. The men were free of known chronic medical conditions at enrollment, and returned for onsite, follow-up visits every 3-5 years. During these visits, detailed physical examinations were performed, bio-specimens including blood were obtained, and questionnaire data pertaining to diet, smoking status, and additional lifestyle factors that may impact health were collected. All participants provided written informed consent to the VA Institutional Review Board (IRB), and both the Harvard T.H. Chan School of Public Health and VA IRBs granted human subjects approval.

All NAS men with continued study participation as of the year 2000, when $PM_{2.5}$ component levels became available, were eligible for our study sample. After excluding participants with a diagnosis of leukemia ($n=11$), due to its potential influence on the DNA methylation of blood cells [30], and those incomplete for the covariates of interest ($n=16$), we had a total of 552 participants with 940 observations between the years 2000 and 2011. Of the 552 participants, 249 (45%) had one visit, 218 (40%) had two visits, and 85 (15%) had three or more visits.

2.2 DNA Methylation and calculation of DNAm-age

Laboratory staff extracted DNA from the buffy coat of whole blood collected from each participant at each NAS follow-up visit (QIAamp DNA Blood Kit, QIAGEN, Valencia, CA, USA). DNA samples were then treated with bisulfite conversion (EZ-96 DNA Methylation Kit, Zymo Research, Orange, CA, USA) and hybridized to the 12 sample Illumina HumanMethylation450 BeadChips (Infinium HD Methylation protocol, Illumina, San Diego, CA, USA). To ensure a similar age distribution and avoid confounding across chips and plates, study staff employed a two-stage age-stratified algorithm to randomize samples. For

quality control, study staff removed samples where >5% of probes had a beadcount < 3 or > 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 [38]. The beta values represent the percentage of methylation for each of the ~480,000 CpG sites in the BeadChip array. The 450k arrays were run in the Genomics Core Facility at Northwestern University.

To explore potential differences in the relationship of PM_{2.5} and PM_{2.5} species with different forms of DNAm-age, we calculated both Horvath DNAm-age and Hannum DNAm-age using the 450k beta values and Horvath's publically available online calculator (<http://labs.genetics.ucla.edu/horvath/dnamage/>). Horvath DNAm-age was derived from an elastic net (penalized regression) using multiple data sets of varying tissue and cell types. 21,369 CpG probes, shared by the Illumina HumanMethylation27 and HumanMethylation450 BeadChip platforms were regressed on a calibrated version of chronological age. The elastic net selected 353 CpGs that correlate with age, and the resulting model coefficients are used by the calculator to predict the age of each DNA sample (DNAm-age) [30]. Hannum DNAm-age was also derived using an elastic net. However, Hannum DNAm-age was based on a single cohort where DNA methylation values were calculated from whole blood. This elastic net selected 71 CpG probes in the Illumina HumanMethylation450 BeadChip that are predictive of chronological age. Hannum DNAm-age was calculated as the sum of the beta values multiplied by the reported effect sizes for the Hannum predictor [36]. The Hannum and Horvath DNAm-ages only share 6 CpG probes (cg04474832, cg05442902, cg06493994, cg09809672, cg19722847, and cg22736354).

2.3 Assessment of Environmental Factors: Ambient Particles and Temperature

We employed the widely used GEOS-chem chemical transport model

(<http://www.geoschem.org>) to generate 1-year exposure estimates for PM_{2.5} and the following major PM_{2.5} component species: organic carbon (OC), elemental carbon (EC), sulfate, nitrate, and ammonium [39]. These 5 component species were selected because they make up a large fraction of total PM_{2.5} mass (~ 88.6%) and were best predicted by the model. GEOS-chem incorporates nonlinear chemistry, meteorology, and detailed emissions inventories to simulate the formation and transportation of atmospheric components to give raw estimates of PM_{2.5} and its major chemical components. Ten-fold cross-validation demonstrated that the model performs well for PM_{2.5} mass and its component species with R²s ranging from 0.70 to 0.88 [40]. We generated daily estimates at the 1x1 km area resolution. Each participant's residence was geocoded and linked to an area level grid-point. Time spent away from home (>7 days) and address changes were also accounted for as particle estimates were assigned to each participants' address. Given that >90% of NAS participants are retired, home address exposures are expected to be a good proxy for their individual ambient exposures. We then generated 1-year total PM_{2.5} and PM_{2.5} component species exposure windows by averaging daily exposures for the 365 days prior to the day of each participants' NAS visit. The 1-year PM_{2.5} exposure window was utilized because it has been previously reported to be robustly associated with DNAm-age [31].

We used a spatiotemporal prediction multi-step approach to generate temperature (in Celsius) for each participant [41]. First, we obtained 1x1 km resolution daily physical surface temperature (Ts) data from NASA satellite measurements and daily near surface air (Ta) data from the Environmental Protection Agency, National Climatic Data Center, and Weather Underground

Inc. We then used mixed model regression to calibrate Ts to Ta. The model was validated with a mean out of sample R^2 of 0.95. To generate 1-year temperature measurements to complement 1-year particle exposures, we averaged daily temperature measurements over the 365 days prior to participants' NAS visits.

2.4 Statistical Analysis

We first used generalized linear mixed effects models to determine the relationship of DNAm-age (Horvath and Hannum independently) with 1-year $PM_{2.5}$ exposure levels and 1-year $PM_{2.5}$ component species exposure levels. All linear mixed effects models included a random participant-specific intercept to account for correlation between repeated measures (*i.e.* multiple visits for a participant).

We adjusted for confounders and covariates that have *a priori* biological/clinical relevance and/or are reported in the existing literature. Specifically, our previous publication was the first study examining associations of ambient particles and DNAm-age [31]. There, we used a tiered approach of adding confounders and covariates based on known relationships of ambient particles with DNA methylation and known relationships of ambient particles with older markers of aging [30, 42-45]. Tier one adjusted for chronological age and blood cell types. Tier two made additional adjustments for lifestyle and environmental factors. Tier three expanded on tier two by additionally adjusting for age-related diseases, and tier four expanded on tier two by additionally adjusting for medications of age-related diseases. After considering model fit (assessed via AIC) and considering biological factors that are known to be important, the tier two covariates were deemed to be most appropriate. Thus, in line with the previously published tier two framework

[31], the models for this analysis were adjusted for chronological age (continuous), six blood cell type estimates [*i.e.* plasma cells, CD4+ lymphocytes, CD8+ lymphocytes, natural killer (NK) cells, monocytes, and granulocytes] (continuous) determined via Houseman and Horvath methods [30, 46], average 1-year temperature (continuous), cumulative cigarette pack years (continuous), smoking status (current, former, or never), 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 ≥ 2 drinks daily), and maximum years of education (continuous). All PM_{2.5} component species models were additionally adjusted for PM_{2.5} mass [47].

To more rigorously identify the PM_{2.5} component species that may be associated with DNAm-age, we applied the adaptive LASSO (least absolute shrinkage and selection operator) [48]. Given that PM_{2.5} component species are correlated, placing them together within the same standard linear regression model can result in unaccounted for stochastic errors. The LASSO is a regression shrinkage and selection approach that helps overcome such limitations. The LASSO applies an l_1 penalty on the component regression coefficients, which minimizes the sum of squared errors subject to the sum of the absolute values of the coefficients being less than a given value [49]. The adaptive LASSO improves upon this procedure by utilizing weights for penalizing different coefficients in the l_1 penalty to identify a subset of model predictors to achieve asymptotic normality [50]. Furthermore, the adaptive LASSO has been successfully applied in air pollution and health research [51, 52].

To identify and select $PM_{2.5}$ component species associated with DNAm-age, we applied a penalty to all $PM_{2.5}$ component species, but not to $PM_{2.5}$ mass and the other covariates in the model. λ , the penalty parameter, determines how strongly the magnitude of the $PM_{2.5}$ species regression coefficients are constrained. When λ is small, the regression coefficients are weakly penalized and mirror those that would be given from a standard linear mixed effects model. When λ is large, the coefficients are strongly penalized, shrinkage is maximized, and all coefficients tend towards zero such that the resulting model includes fixed covariates only. When λ takes a value in between the extremes, the result is a penalized model where some $PM_{2.5}$ component species will have coefficients of zero and others will be non-zero. $PM_{2.5}$ component species with non-zero coefficients are considered as “selected” by the adaptive LASSO. We ran the model across a range of λ s, beginning with a λ of 0, and selected the λ resulting in the model with the smallest Bayesian Information Criterion (BIC) [53]. Following LASSO selection, we fit a final multiple-species linear mixed effects model using the selected $PM_{2.5}$ component species and our fixed covariates. From this final model, we were able to estimate component species effect sizes and their corresponding 95% confidence intervals.

Additionally, we considered that the LASSO may not select the $PM_{2.5}$ species that are most correlated with total $PM_{2.5}$ mass. Thus, we conducted a sensitivity analysis where we performed LASSO selection without adjusting for $PM_{2.5}$ mass. From this sensitivity analysis model, we fit a multiple-species linear mixed effects model using the selected $PM_{2.5}$ component species and estimated component species effect sizes and their corresponding 95% confidence intervals.

After finding that Horvath DNAm-age alone was significantly associated with PM_{2.5} component species, we evaluated the relationships of the DNA methylation values of each of the 353 Horvath CpG probes with the particles in the aforementioned LASSO-selected multiple-species linear mixed effects model. In addition to the already described covariates, we included technical covariates (450k plate, chip, row, and column) to this analysis. To account for multiple hypothesis testing, we also performed FDR correction in this analysis. We then performed gene ontology analysis on the list of significant CpGs (FDR P-value < 0.05) using the publically available GoTermFinder tool (<http://go.princeton.edu/cgi-bin/GOTermFinder>).

In an additional sensitivity analyses, we re-ran our models excluding participant visits with PM_{2.5} exposures greater than 12 µg/m³. This allowed us to assess if our findings persisted even at the PM_{2.5} levels currently deemed acceptable by the U.S. Environmental Protection Agency (EPA) National Ambient Air Quality Standards (NAAQS) [54].

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

3 | Results

3.1 Descriptive Results

Table 1 summarizes the characteristics of the study population. All study participants were Caucasian males with a mean (\pm SD) age of 74.7 ± 6.99 years across all study visits. Average Horvath DNAm-age and Hannum DNAm-age were 74.0 ± 7.92 years and 75.1 ± 8.95 years respectively. Horvath DNAm-age ($r = 0.59$, $p < 0.0001$) and Hannum DNAm-age ($r = 0.77$,

p<0.0001) were both strongly correlated with chronological age in the study population. Both measures of DNAm-age were also strongly correlated to each other ($r = 0.69$, $p<0.0001$).

Table 1. Characteristics of Study Subjects (2000 – 2011)

Variable	First Visit (N = 552)	All Visits (N = 940)
Age (years), mean (SD)	73.3 (6.82)	74.7 (6.99)
Horvath DNAm-age (years), mean (SD)	73.7 (7.77)	74.0 (7.92)
Hannum DNAm-age (years), mean (SD)	73.8 (8.80)	75.1 (8.95)
Temperature (°C), mean (SD)	11.5 (1.12)	11.3 (1.00)
Pack years, mean (SD)	20.7 (24.7)	20.5 (24.4)
Smoking Status, N (%)		
Current	25 (4)	40 (4)
Former	355 (64)	614 (65)
Never	172 (32)	286 (31)
Season, N (%)		
Spring	145 (26)	241 (26)
Summer	115 (21)	199 (21)
Fall	177 (32)	313 (33)
Winter	115 (21)	187 (20)
BMI, N (%)		
Healthy/Lean	119 (21)	216 (23)
Overweight	302 (55)	493 (52)
Obese	131 (24)	231 (25)
Alcohol Consumption, N (%)		
< 2 drinks/day	441 (80)	761 (81)
≥ 2 drinks/day	111 (20)	179 (19)
Education, N (%)		
≤ 12 years	146 (27)	242 (26)
12 - 16 years	262 (47)	434 (46)
> 16 years	144 (26)	264 (28)

Table 2 reports 1-year PM_{2.5} and PM_{2.5} component species exposure levels across all study visits.

The participants had a mean 1-year PM_{2.5} exposure level of $10.3 \pm 1.60 \mu\text{g}/\text{m}^3$, with an interquartile range (IQR) of $2.16 \mu\text{g}/\text{m}^3$. Of the measured PM_{2.5} component species, sulfate

accounted for the largest proportion of total PM_{2.5} mass (33%), followed by organic carbon (28.6%), nitrate (11.5%), ammonium (10.1%), and elemental carbon (5.4%). OC was the PM_{2.5} species most correlated with total PM_{2.5} mass ($r = 0.67$). 1-year PM_{2.5} and PM_{2.5} species Pearson correlations across all visits are reported in Table S1. Moreover, 1-year PM_{2.5} and PM_{2.5} species exposure distributions across first visits are reported in Table S2.

Table 2. Mean 1-Year Particulate Matter 2.5 (PM_{2.5}) and Component Species Concentrations Across All Study Visits

Particle (µg/m ³)	Mean (SD)	IQR	Proportion of PM _{2.5} (%)	Pearson Correlation with PM _{2.5}	N
PM _{2.5}	10.3 (1.60)	2.16	-	-	940
PM _{2.5} Component Species					
EC	0.56 (0.17)	0.23	5.4	0.62	940
OC	2.94 (0.91)	1.28	28.6	0.67	940
Sulfate	3.40 (1.23)	0.82	33.0	0.30	940
Nitrate	1.18 (0.32)	0.42	11.5	0.46	940
Ammonium	1.04 (0.31)	0.3	10.1	0.53	940

3.2 1-Year PM_{2.5} and PM_{2.5} Component Species as Predictors of DNAm-age

Table 3 summarizes the results of three model frameworks where PM_{2.5} and its component species were modeled as predictors of both Horvath and Hannum DNAm-age. Residuals from all models appeared normally distributed. In the model framework 1, PM_{2.5} was modeled as a predictor of Horvath and Hannum DNAm-age independently. In the fully adjusted model, an IQR increase in 1-year PM_{2.5} exposure was significantly associated with a 0.64-year increase in Horvath DNAm-age ($p=0.005$). However, an IQR increase in 1-year PM_{2.5} exposure was not significantly associated with Hannum DNAm-age ($\beta=0.06$, $p=0.74$).

Under the model framework 2, each PM_{2.5} component species was modeled as an independent predictor of Horvath and Hannum DNAm-age adjusting for all covariates and total PM_{2.5} mass. 1-year IQR increases in OC ($\beta=0.93$, $p=0.001$), sulfate ($\beta=0.59$, $p<0.0001$), nitrate ($\beta=0.58$, $p=0.01$), and ammonium ($\beta=0.59$, $p=0.0004$) were all significantly associated with increases in Horvath DNAm-age of at least 0.58 years. No PM_{2.5} component species were significantly associated with Hannum DNAm-age (Table 3).

The model 3 framework reflects the results of the multiple-species fully-adjusted linear mixed effects models with the PM_{2.5} component species selected by the adaptive LASSO. The adaptive LASSO selected sulfate and ammonium as important predictors of Horvath DNAm-age. Figure 1A depicts the relationship between BIC, the model selection criterion, and λ , the adaptive LASSO penalty parameter. The model with the smallest BIC had $\lambda = 11$. Figure 1B shows the LASSO coefficient paths for the PM_{2.5} component species. Each component species coefficient is expressed as the difference in mean Horvath DNAm-age per an IQR increase in the 1-year component species exposure level. Each curve depicts the rate at which the component species coefficient shrinks towards zero as λ increases. At $\lambda=0$, all components species have a non-zero coefficient.

Table 3. 1-Year Particulate Matter 2.5 (PM_{2.5}) and Component Species as Predictors of DNA Methylation (DNAm) Age

Particle	Difference in Horvath DNAm-age for IQR (95% CI)	<i>P</i>	Difference in Hannum DNAm-age for IQR (95% CI)	<i>P</i>	N
Model Framework 1					
PM _{2.5}	0.64 (0.20, 1.09)	0.005	0.06 (-0.28, 0.40)	0.74	940
Model Framework 2					
EC	0.27 (-0.25, 0.80)	0.30	-0.09 (-0.48, 0.29)	0.64	940
OC	0.93 (0.37, 1.50)	0.001	0.35 (-0.05, 0.77)	0.09	940
Sulfate	0.59 (0.37, 0.81)	<0.0001	0.08 (-0.09, 0.25)	0.36	940
Nitrate	0.58 (0.11, 1.04)	0.01	0.30 (-0.04, 0.65)	0.08	940
Ammonium	0.59 (0.26, 0.92)	0.0004	0.06 (-0.18, 0.30)	0.63	940
Model Framework 3					
PM _{2.5}	0.18 (-0.30, 0.66)	0.45	-	-	940
Sulfate	0.51 (0.28, 0.74)	<0.0001	-	-	940
Ammonium	0.36 (0.02, 0.70)	0.04	-	-	940

Model Framework 1: adjusted for chronological age, blood cell types, temperature, pack years, smoking status, season, BMI, alcohol consumption, and education. *Model Framework 2:* PM_{2.5} component species as independent predictors of DNAm-age adjusted for PM_{2.5} in addition to model 1 covariates. *Model Framework 3:* PM_{2.5}, sulfate, and ammonium as joint predictors of DNAm-age (given selection of sulfate and ammonium by the adaptive LASSO) adjusted for model 1 covariates. No species were selected as predictors of Hannum DNAm-age.

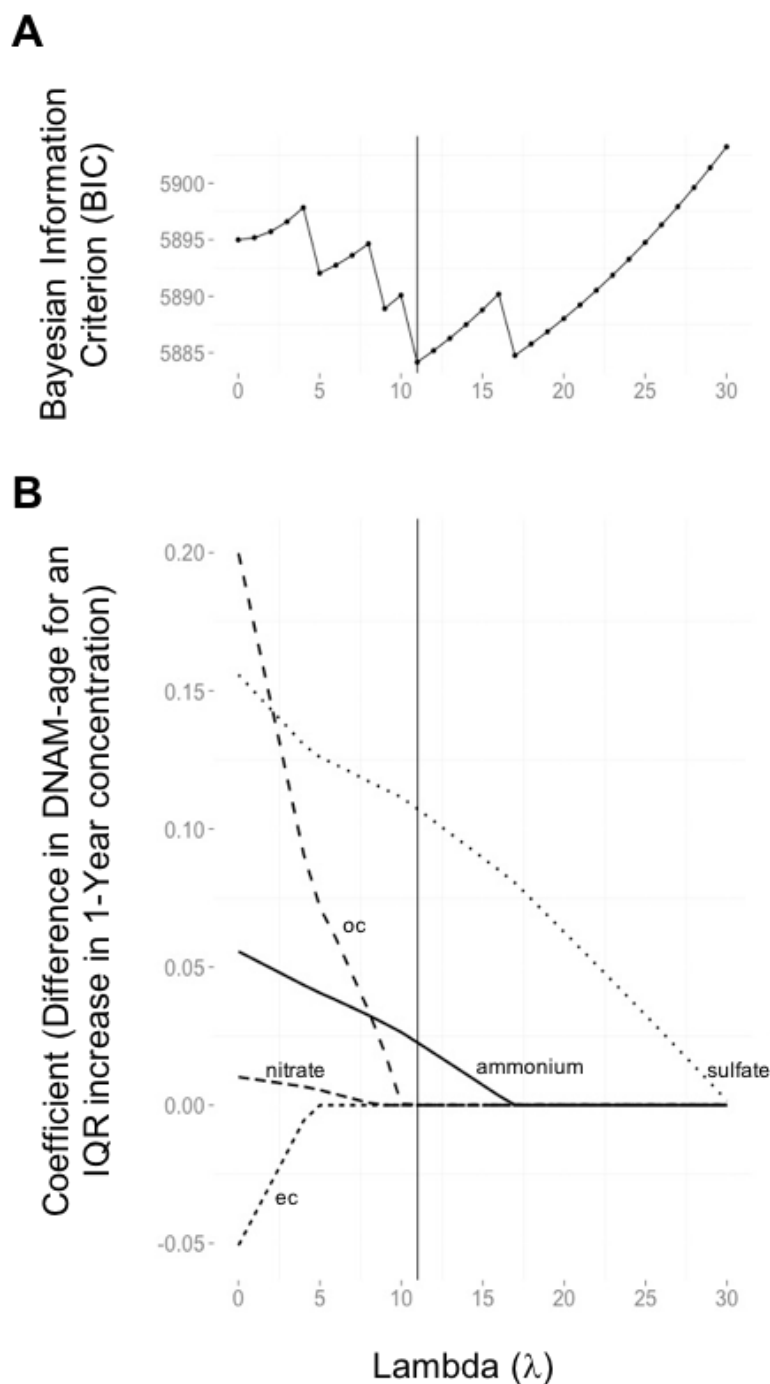


Figure 1 | A) The relationship between BIC, a criterion for model selection and λ (lambda), the adaptive LASSO penalty parameter, for DNAM-age. The vertical line at $\lambda = 11$ denotes the penalty parameter with the lowest BIC. B) LASSO coefficient paths: plot of coefficient profiles for PM_{2.5} components as a function of λ . At $\lambda = 11$, sulfate and ammonium are the only PM_{2.5} components with a non-zero coefficient.

In the multiple-species fully-adjusted linear mixed effects model, both sulfate ($\beta=0.51$, $p<0.0001$) and ammonium ($\beta=0.36$, $p=0.04$) remain significant positive predictors of Horvath DNAm-age. The adaptive LASSO did not select any $PM_{2.5}$ component species as important predictors of Hannum DNAm-age.

In our sensitivity analysis – where LASSO selection was performed without adjusting for total $PM_{2.5}$ mass – sulfate, ammonium, and OC were selected as important predictors of DNAm-age (Figure S1). Nonetheless, in a multiple-species fully-adjusted linear mixed effects model, both sulfate ($\beta=0.45$, $p=0.0003$) and ammonium ($\beta=0.34$, $p<0.05$) remained significant positive predictors of Horvath DNAm-age, but OC ($\beta=0.42$, $p=0.16$) was not a significant predictor of Horvath DNAm-age (Table S3). Again, the sensitivity analysis adaptive LASSO did not select any $PM_{2.5}$ component species as important predictors of Hannum DNAm-age.

Significant findings from the main analysis multiple-species fully-adjusted linear mixed effects model persisted in the second sensitivity analyses excluding participant visits with $PM_{2.5}$ exposures greater than $12 \mu\text{g}/\text{m}^3$, the annual $PM_{2.5}$ exposure level currently deemed acceptable by the U.S. Environmental Protection Agency (EPA) National Ambient Air Quality Standards (NAAQS) (Table S4).

3.3 Associations between 1-Year $PM_{2.5}$ and $PM_{2.5}$ Component Species Levels and Methylation Values at Horvath DNAm-age CpG Sites

After FDR correction, 47 out of 353 Horvath DNAm-age CpG sites had methylation values that were significantly associated with total $PM_{2.5}$ levels in the fully-adjusted multiple-species linear

mixed effects model. PM_{2.5} levels were positively or negatively associated with CpG methylation values depending on the CpG site (Table 4). 46 of the 47 CpG sites mapped to known genes. 9 of these 46 genes (*ENPP2*, *FAM50B*, *LZTFL1*, *SGCE*, *C14orf105*, *ZBTB5*, *TMEM132E*, *CATSPERG*, and *NDUFA13*) were previously reported in a similar, previously published PM_{2.5} Horvath CpG analysis [31]. Gene ontology of our 46 genes combined with the genes in the previously reported study returned the GO term “regulation of translational initiation” (Table S5).

Only 1 out 353 CpG sites (cg02275294) had methylation values that were significantly associated with ammonium levels in the fully-adjusted multiple-species linear mixed effects model. No individual CpG sites had methylation values that were significantly associated with sulfate levels after FDR correction.

Table 4. 1-Year Particle Exposures as Predictors of Horvath CpG Probe

CpG	Gene	Process/Function	Difference in Methylation per SD (%)	Direction of Association	FDR Adjusted <i>P</i>
PM_{2.5}					
cg15262928	TIMM17A	Mitochondrial protein import	24.18	+	0.001
cg14409958	ENPP2*	Nucleic acid binding	19.85	+	0.001
cg01570885	FAM50B*	Protein binding	19.64	-	0.004
cg08186124	LZTFL1*	Protein binding: cytoplasm	19.59	+	0.004
cg18139769	SGCE*	Calcium binding	19.25	-	0.004
cg15547534	PPP1R35	Phosphatase binding	18.79	+	0.004
cg26456957	PPP1R12C	Protein kinase binding	18.74	+	0.001
cg05847778	BBS5	Transcription initiation	18.01	+	0.006
cg15661409	C14orf105*	Uncharacterized	17.52	-	<0.001
cg02335441	NEK11	DNA replication	17.32	+	0.008

cg17285325	TYMP	Phosphorylase activity	17.23	+	0.007
cg04094160	ZBTB5*	Transcriptional regulation	16.92	+	0.003
cg03682823	SGCE	Calcium binding	16.80	-	0.008
cg07663789	NPR3	Hormone binding/blood volume	16.13	+	0.003
cg15703512	PDZD9	Uncharacterized	15.80	+	0.015
cg22190114	NLRP8	ATP binding	15.60	+	0.015
cg19008809	SFMBT1	Transcription corepressor activity	15.48	+	0.013
cg00374717	ARSG	Sulfatase enzyme activity	15.29	-	0.004
cg12985418	MIB1	Protein binding	15.14	+	0.018
cg03588357	GPR68	G-protein coupled receptor activity	15.09	+	0.020
cg14424579	AGBL5	Metalloproteinase	15.07	+	0.007
cg14597908	GNAS	G-protein binding	15.04	-	0.015
cg19044674	LEPRE1	Oxidoreductase activity	14.88	+	0.023
cg09441152	PQLC1	Membrane component	14.87	+	0.027
cg07849904	MN1	Transcriptional activator	14.85	+	0.015
cg19273182	PAPOLG	Polynucleotide adenylyltransferase activity	14.81	+	0.025
cg17063929	NOX4	Nucleotide binding	14.56	-	0.015
cg24116886	DEFB127	Immunologic response	14.40	-	0.015
cg09191327	PRDM12	Methyltransferase activity	14.30	+	0.027
cg23662675	ZMYND8	Transcription cofactor activity	14.16	+	0.014
cg14992253	EIF3I	Translation initiation	13.12	-	0.018
cg05442902	P2RX6	Channel activity	12.82	-	0.026
cg06557358	TMEM132E*	Integral component of membrane	12.60	+	0.031
cg11932564	TNFRSF13C	Immunologic response	12.51	+	0.037
cg18031008	MRPS21	Mitochondrial ribosome	12.10	+	0.030
cg19945840	SDF4	Calcium binding	12.08	-	0.023
cg19167673	PDGFB	Protein homodimerization activity	12.03	+	0.031
cg25159610	PLK2	Cell division	11.96	+	0.038
cg22006386	CATSPERG*	Ion channel activity	11.89	+	0.026
cg27377450	<i>unknown</i>	<i>unknown</i>	11.85	-	0.026
cg20100381	NAE1	Protein heterodimerization activity	11.84	+	0.046
cg04268405	CHST3	Sulfotransferase	11.47	-	0.026
cg07595943	ADAD2	RNA binding	11.36	-	0.023
cg25505610	EIF3M	Translation initiation	10.67	+	0.042

cg16744741	PRKG2	Protein kinase activity	10.17	-	0.038
cg21395782	NDUFA13*	NADH dehydrogenase activity	8.27	+	0.027
cg01459453	SELP	Oligosaccharide binding	8.05	-	0.023

Ammonium

cg02275294	SOAT1	Fatty-acyl-CoA binding	10.81	+	0.036
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All models are fully adjusted. * = CpGs associated with PM_{2.5} levels in a prior publication.

4 | Discussion

In this study, we report positive associations of 1-year PM_{2.5} exposure levels with Horvath DNAm-age in a population of community-dwelling, elderly men. Additionally, we utilized the adaptive LASSO to identify 1-year sulfate and ammonium levels as the PM_{2.5} components most robustly associated with Horvath DNAm-age. To our knowledge, this is the first report of associations of multiple PM_{2.5} component species with DNAm-age and the second time that satellite-derived PM_{2.5} exposure levels have been found to be associated with Horvath DNAm-age. In addition to being consistent with the existing literature [31], our findings also demonstrate important public health relevance as they persist in sensitivity analyses including only participant visits with 1-year PM_{2.5} levels within current US EPA national ambient air quality standards [54]. Our study also extends the literature by exploring PM_{2.5} relationships with Hannum DNAm-age although these relationships were found to be null. Furthermore, we identified 47 CpG sites, 9 of which were previously reported, whose methylation values were significantly associated with PM_{2.5} levels in fully-adjusted linear mixed effects models. Only 1 CpG was associated with ammonium levels and 0 were associated with sulfate levels.

Given our prior report of robust associations of $PM_{2.5}$ levels from satellite-based spatiotemporal models with Horvath DNAm-age, we expected to observe a similar positive relationship using $PM_{2.5}$ levels from the GEOS-chem chemical transport model. As expected, we observed that an IQR increase in 1-year $PM_{2.5}$ exposure was associated with a 0.64-year increase in Horvath DNAm-age. Since $PM_{2.5}$ component species are highly related to total $PM_{2.5}$, we also expected that $PM_{2.5}$ component species would be associated with Horvath DNAm-age, even when adjusting for $PM_{2.5}$ mass. Given the existing literature concerning the differential health effects of $PM_{2.5}$ component species, we speculated that some component species may be more robustly associated with Horvath DNAm-age than others. In particular, we expected the carbonaceous fractions to be among the species most robustly associated with DNAm-age due to the extensive literature (including work from our group) on the adverse nature of carbonaceous fraction exposures on health [31, 43, 55-57]. In our fully adjusted one-species linear mixed effects models, we observed strong positive associations of 4 out of the 5 component species examined with Horvath DNAm-age. IQR range increases in organic carbon, sulfate, nitrate, and ammonium were all significantly associated with at least a 0.58-year increase in Horvath DNAm-age.

Despite the results from our fully adjusted one-species linear mixed effects models, we desired a method to more comprehensively identify the component species most associated with DNAm-age. Nevertheless, we were aware that simply modeling highly-correlated $PM_{2.5}$ species together would result in unaccounted for stochastic errors. Thus, we employed the adaptive LASSO as a penalized regression method to help overcome this difficulty. The literature has shown that

carbonaceous fractions are robustly associated with age-related health outcomes [31, 43, 55-57]; however, neither elemental or organic carbon were selected in our models. Rather sulfate and ammonium were selected. This difference may be explained by the fact that a majority of the aforementioned studies did not consider other PM_{2.5} component species in addition to the carbonaceous fractions. Even in our single-species linear mixed effects models, we note that organic carbon was among the four species significantly associated with Horvath DNAm-age (Table 3). However, when all five component species are considered together in the adaptive LASSO, only sulfate and ammonium were selected. It is also possible that the LASSO did not select the carbonaceous fractions because the selection was performed under PM_{2.5} adjustment and PM_{2.5} may be capturing most of the variability of organic and elemental carbon. Thus, we performed LASSO selection not adjusting for total PM_{2.5} mass as a sensitivity analysis. This time LASSO did select organic carbon along with sulfate and ammonium. However, when these three component species were modeled with PM_{2.5} in a multiple-species fully-adjusted linear mixed effects model, organic carbon was the only species that was not a significant predictor of DNAm-age. This suggests that organic carbon was selected in the sensitivity analysis because of its strong correlation with PM_{2.5} mass and not because organic carbon itself is a good predictor of DNAm-age. This finding also reiterates the notion that adjustment for PM_{2.5} mass in component species models is very important as PM_{2.5} mass often confounds the relationship between the outcome and species [47]. Failing to include PM_{2.5} mass may lead to misleading findings about species. In all, our data suggests that of the considered species, sulfate and ammonium have the most important relationships with DNAm-age. Furthermore, existing studies that do consider a range of PM_{2.5} components demonstrate that other non-carbonaceous components are important to age-related outcomes [52, 58, 59]. These data, together with our findings, also suggests the

important need to consider a range of $PM_{2.5}$ components, rather than one or two species, in air pollution and health studies.

Both sulfate and ammonium are classified in the inorganic fraction of $PM_{2.5}$. Sulfates are often produced from oxidation or photochemical reactions involving primary gases derived from sources like coal-burning power plants [60]. Additionally, ammonia from organic sources including animal feeds and fertilizers can contribute to the existence of sulfates in the form of atmospheric ammonium sulfate [61]. As far as direct ambient sulfate and ammonium toxicity to human health is concerned, existing studies are limited. Yet, there has been extensive evidence describing the ability of acidic sulfates, like ammonium sulfate, to increase the number and toxicity of biologically harmful secondary particles [47, 62-66]. For instance, ammonium sulfate aerosols have been shown to influence the photo-chemical reactions of nitrogen oxides and toluene hastening the production of secondary organic aerosols [67]. Moreover, sulfur concentrations have been found to be directly proportional to the ability of soluble particle extracts to generate biologically damaging oxidants [68]. Furthermore, a prior study in the NAS has reported a 27% decrease in long interspersed nucleotide element-1 methylation per every IQR increase in 90-day sulfate exposure. This study provides evidence for the influence of sulfates on DNA methylation, which may be a potential pathway for sulfate toxicity [42]. It is still unclear what the molecular relevance of Horvath DNAm-age is, but our findings along with the existing literature will be helpful in providing additional insight for future work.

Following the selection of sulfate and ammonium by the adaptive LASSO, we constructed a final multiple-species linear mixed effects model adjusted for $PM_{2.5}$ mass and all covariates. Even in

this model, sulfate and ammonium remained significant positive predictors of Horvath DNAm-age. We then looked to see if there were specific Horvath DNAm-age component CpG sites with methylation values that were associated with PM_{2.5}, sulfate, and/or ammonium in our fully-adjusted multiple-species linear model. From this analysis, we identified 47 significant CpG sites after FDR adjustment. These sites mapped to 46 genes, and 9 of them were reported in a previous CpG-level analysis of the same 353 sites in the Horvath algorithm that we conducted using PM_{2.5} levels from a satellite-based spatiotemporal model. To better grasp the impact of PM_{2.5} levels on methylation, we divided the coefficients for each significant CpG site (*i.e.* difference in methylation per IQR increase in particle level) by the standard deviation of the respective particle level. We were pleased to see that 5 of the 9 CpGs that were shared between both PM_{2.5} prediction models were in the top 20% of our gene list. We then combined the gene lists from both PM_{2.5} prediction models (removing any duplicates) and performed a gene ontology (GO) analysis. The GO analysis returned the term “regulation of translational initiation” with the following genes from our list falling into this category: RXRA, EIF3M, EIF31. Though the GO term itself is not highly specific, combining this pathway with what is known about the toxicity of PM_{2.5} will be useful in further understanding how PM_{2.5} may contribute to aging and disease. Only 1 CpG was associated with ammonium levels and it mapped to the gene SOAT1, which is involved in fatty-acyl-CoA binding. SOAT1 has been implicated in a number of diseases including familial hypercholesterolemia [69]. No CpG sites were specifically associated with sulfate levels. The finding that almost no CpGs sites were associated with ammonium and sulfate further demonstrates that Horvath DNAm-age is simply not a reflection of its 353 component CpGs, and reiterates the need for work focused on defining the molecular relevance of DNAm-age.

Finally, our study demonstrates that all DNAm-age measures are not the same. In the literature there is evidence of both Horvath and Hannum DNAm-age reflecting the same disease outcome and evidence where they differ in their reporting ability. For instance, both Horvath and Hannum DNAm-age appear to be useful in predicting mortality [70, 71]. However, in a study of male and female veterans, Hannum DNAm-age was associated with post-traumatic stress disorder and neural integrity, but Horvath DNAm-age was not [72]. The differences in these two DNAm-age measures may stem from the fact that they are derived from almost entirely different CpG sites or from the fact that Horvath DNAm-age was constructed using many datasets of multiple tissue types and the Hannum DNAm-age was based only on blood from one dataset [30, 36]. Our results suggest that Hannum DNAm-age is not sensitive to exposure levels of PM_{2.5} and its component species. Additional studies in different populations will be necessary to confirm these findings more broadly. Nonetheless, continued research exploring the specific sensitivity of DNAm-age measures will be a crucial next step in the growth of this field of research. Once more is known about the profiles of these markers, we can begin to use them more effectively in answering questions concerning human health.

Strengths of our study include rigorous statistical methods and access to a large cohort with extensive and repeated information regarding pollutant exposures, potential confounders, and DNA methylation data from multiple study visits. However, our study does have several limitations. First, although we used a validated chemical transport model to estimate the levels of ambient PM_{2.5} and its component species at participants' addresses, we recognize that these estimates may differ from personal exposures. Nonetheless, we know that a majority of NAS

participants are retired and spend most of their time at home. Moreover, our approach is expected to result in non-differential misclassification that is likely to underestimate the observed associations rather than bias them away from the null [73]. Secondly, it is known that LASSO regression is limited to linear relationships. Given the linear relationship of our particle exposures with DNAm-age and the scope of this paper, the adaptive LASSO was a good tool for identifying PM_{2.5} components that are independently important to DNAm-age. However, for future studies potentially interested in the interactions between PM_{2.5} components, another technique may be necessary as PM_{2.5} species interactions that are important for the prediction of DNAm-age may be more complex (*i.e.* not linear). Third, we note that our findings are based on an elderly cohort of Caucasian males that reside in a lightly-polluted environment. Hence, additional studies involving other demographic groups and in different environments will be necessary to confirm our findings more broadly. Finally, we used the existing literature and *a priori* knowledge of biological/clinical relevance to adjust for potential confounders. Nonetheless, we cannot rule out the possibility of unknown or residual confounding in our analyses.

5 | Conclusion

Our study utilizes the GEOS-chem chemical transport model to [validate](#) novel positive associations between long-term PM_{2.5} exposure levels and Horvath DNAm-age. For the first time, we demonstrate that sulfate and ammonium are among the PM_{2.5} component species most associated with Horvath DNAm-age in this population of elderly men. In contrast, we observed no relationships of long-term PM_{2.5} and PM_{2.5} component species exposure levels with Hannum DNAm-age. These results suggest that DNA methylation-based biomarkers of age differ in their

sensitivity to ambient particle exposures and potentially disease outcomes. Future studies in other populations will be critical for defining the environmental and disease sensitivity profiles of DNAm-age measures.

Contributors: JCN and JDS conceived and designed the study. EC, QD, IK, ACJ, LH, and PV gathered data. JCN performed the data analyses and drafted the manuscript. LD, JDS, EC, YO, MGW, and AAB contributed to the analyses. All authors revised and approved the manuscript.

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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).

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