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Research article

Modifying Role of Endothelial Function Gene Variants on the Association of Long-Term $PM_{2.5}$ Exposure With Blood DNA Methylation Age: The VA Normative Aging Study

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ABSTRACT

Recent studies have reported robust associations of long-term $PM_{2.5}$ exposure with DNA methylation-based measures of aging; yet, the molecular implications of these relationships remain poorly understood. We evaluated if genetic variation in 3 biological pathways implicated in $PM_{2.5}$-related disease—oxidative stress, endothelial function, and metal processing—could modify the effect of $PM_{2.5}$ on DNAm-age, one prominent DNA methylation-based measure of biological age. This analysis was based on 552 individuals from the Normative Aging Study with at least one visit between 2000 and 2011 (n = 940 visits). A genetic-score approach was used to calculate aging-risk variant scores for endothelial function, oxidative stress, and metal processing pathways. One-year $PM_{2.5}$ and $PM_{2.5}$ component (sulfate and ammonium) levels at participants' addresses were estimated using the GEOS-chem transport model. Blood DNAm-age was calculated using CpG sites on the Illumina HumanMethylation450 BeadChip. In fully-adjusted linear mixed-effects models, the effects of sulfate on DNAm-age (in years) were greater in individuals with high aging-risk endothelial function variant scores when compared with individuals with low aging-risk endothelial function variant scores ($P_{interaction} = 0.0007$; $\beta_{High} = 1.09$, 95% CI_{High}: 0.70, 1.48; $\beta_{Low} = 0.40$, 95% CI_{Low}: 0.14, 0.67). Similar trends were observed in fully adjusted models of ammonium and total $PM_{2.5}$ alone. No effect modification was observed by oxidative stress and metal processing variant scores. Secondary analyses revealed significant associations of serum endothelial markers, intercellular adhesion molecule-1 ($\beta = 0.01$, 95% CI: 0.002, 0.012) and vascular cell adhesion molecule-1 ($\beta = 0.002$, 95% CI: 0.0005, 0.0026), with DNAm-age. Our results add novel evidence that endothelial physiology may be important to DNAm-age relationships, but further research is required to establish their generalizability.

Key words: DNA methylation age; particulate matter 2.5; endothelial function.

Approximately 92% of the world's population lives in areas with adverse ambient fine particle ($PM_{2.5}$) levels (Mayor, 2016). In fact, a substantial portion of global mortality and morbidity can be attributed to $PM_{2.5}$ exposure (Apte *et al.*, 2015). Research continues to demonstrate that long-term $PM_{2.5}$ exposure is a major risk factor for cardiovascular disease (Martinelli *et al.*, 2013; Zhong *et al.*, 2015), and respiratory impairment (Chen *et al.*, 2017). $PM_{2.5}$ has also been associated with cognitive decline (Power *et al.*, 2011; Schikowski *et al.*, 2015), and cancer (Lepeule *et al.*, 2012; Raaschou-Nielsen *et al.*, 2016). Still, exactly how $PM_{2.5}$ contributes to these and other important health outcomes is not fully understood. Addressing this knowledge gap is a critical step for developing interventions to alleviate the disease burden of individuals already exposed to harmful $PM_{2.5}$ levels. Aging also independently contributes to many $PM_{2.5}$-related health endpoints (Lane-Cordova *et al.*, 2016; Rowe and Kahn, 2015). Thus, studying how $PM_{2.5}$ is related to aging may facilitate a greater understanding of the complex pathophysiology surrounding $PM_{2.5}$-related diseases.

Some of the most recent studies of $PM_{2.5}$ and aging have involved DNA methylation age (DNAm-age), a novel tissue-independent measure of biological age that is calculated using DNA methylation values from 353 age-correlated CpG dinucleotides (Horvath, 2013). Our research group was the first to report significant positive associations of long-term $PM_{2.5}$ exposure levels with DNAm-age (Nwanaji-Enwerem *et al.*, 2016). Recently, we have identified sulfate and ammonium as important component species in the $PM_{2.5}$-DNAm-age relationship (Nwanaji-Enwerem *et al.*, 2017). In collaboration with another research group, we have also demonstrated associations between other air pollutants (eg, black carbon, PM_{10}, and NO_x) and epigenetic aging measures (Ward-Caviness *et al.*, 2016). Despite this work, very little is known about the molecular implications of the $PM_{2.5}$ and DNAm-age relationship. At the moment, the algorithm used to calculate DNAm-age relies on assays that have only been optimized for humans and chimpanzees (Horvath, 2013). Hence, this field of research is limited by a lack of traditional animal models to study DNAm-age's relationships. It is possible that DNAm-age is simply reflecting a well-studied biological process (eg, oxidative stress), but it may alternatively reflect a completely novel process. Existing epidemiologic studies have demonstrated robust associations of DNAm-age with mortality, cognitive decline, cancer, and other $PM_{2.5}$-related outcomes thereby suggesting that DNAm-age processes are common to many disease pathologies (Chen *et al.*, 2016; Levine *et al.*, 2015; Wolf *et al.*, 2016). There is also epidemiologic evidence that normal genetic variation may influence DNAm-age, but this has yet to be explored in the context of the $PM_{2.5}$-DNAm-age association (Horvath *et al.*, 2016). To our knowledge, only a couple of studies have peripherally examined the molecular implications of DNAm-age, and these studies only conclude that DNAm-age represents a form of biological aging that differs from cellular senescence (Horvath, 2013; Lowe *et al.*, 2016).

Even with current limitations, existing technologies can be creatively utilized to begin understanding the molecular implications of DNAm-age. Here, we employ components of a previously developed genetic score approach that categorizes normal genetic variation into 3 biological pathways—oxidative stress, endothelial function, and metal processing (Bind *et al.*, 2014). Oxidative stress, endothelial function, and metal processing are all biological pathways that have been implicated in numerous $PM_{2.5}$-related diseases; thus, they may also be involved in the $PM_{2.5}$-DNAm-age relationship (Dai *et al.*, 2016). The existing studies that have used this Bind *et al.* (2014) method have suggested potential modification of the associations of $PM_{2.5}$ exposure with inflammatory markers and cardiac autonomic function, but the results have not been statistically significant (Dai *et al.*, 2016; Mordukhovich *et al.*, 2015, 2016). Given these previous findings, our aim is to use the candidate pathway-specific genetic variants employed by the Bind method to (1) identify variants specifically important to DNAm-age and (2) assess if the normal genetic variation captured by these variants modifies the $PM_{2.5}$-DNAm-age relationship in a population of community-dwelling elderly Caucasian men.

MATERIALS AND METHODS

Study Population

Participants included in this analysis were part of the Normative Aging Study (NAS), an ongoing longitudinal cohort study of healthy male volunteers from the Eastern Massachusetts area (Bell *et al.*, 1966). The NAS is a closed cohort and participants are now elderly. The NAS was established by the U.S. Department of Veterans Affairs (VA) in 1963, and enrolled men who were free of any chronic disease. Every 3–5 years, NAS participants reported for onsite, detailed medical examinations during which bio-specimens were collected and assessments of lifestyle factors that may affect health were made. All participants provided written informed consent to the VA Institutional Review Board (IRB), and human subject's approval was granted by the VA and Harvard T.H. Chan School of Public Health IRBs.

All NAS men with continued study participation as of the year 2000, when address-specific $PM_{2.5}$ component species 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 (Horvath, 2013), and those incomplete for the covariates of interest (n = 16), we had a total of 552 participants with 940 observations (ie, study visits) between the years 2000 and 2011. This was the study sample that was used in reporting the significant associations between $PM_{2.5}$ component species and DNAm-age in our previous publication (Nwanaji-Enwerem *et al.*, 2017). Of these 552 participants, 249 (45%) had 1 visit, 218 (40%) had 2 visits, and 85 (15%) had 3 or more visits. From this sample, we then excluded participants missing pathway specific polymorphism data. This resulted in 3 distinct, but not mutually exclusive, groups of participants: (1) Oxidative stress subset (n = 410, obs = 702); (2) Endothelial function subset (n = 450, obs = 779); and (3) Metal processing subset (n = 426, obs = 744).

DNA Methylation Assay and Calculation of DNAm-Age

Laboratory staff extracted DNA from the buffy coat of whole blood collected from each participant during each NAS follow-up visit (QIAamp DNA Blood Kit, QIAGEN, Valencia, California, USA). DNA samples underwent bisulfite conversion (EZ-96 DNA Methylation Kit, Zymo Research, Orange, California, USA) and were hybridized to the 12 sample Illumina HumanMethylation450 BeadChips (Infinium HD Methylation protocol, Illumina, San Diego, California, USA). A 2-stage age-stratified algorithm was used to randomize samples avoiding confounding and ensuring a similar age distribution across chips and plates. For quality control purposes, study staff removed samples where >5% of probes had a beadcount < 3 or > 1% of probes had a detection *P*-value > .05. The Bioconductor minfi package Illumina-type background correction without normalization was used to preprocess the remaining samples

and generate methylation beta values (Aryee *et al.*, 2014). Beta values represent the percentage of methylation for each of the approximately 480 000 CpG sites in the BeadChip array. The 450 k arrays were run in the Genomics Core Facility at Northwestern University.

DNAm age was determined using the publically available online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/; last accessed April 29, 2017). DNAm-age was derived from penalized regression (an elastic net) using multiple datasets of varying cell and tissue 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 correlated with age (193 positively and 160 negatively), and the resulting model coefficients were used by the calculator to predict the age of each DNA sample (DNAm-age) (Horvath, 2013). Empirical data demonstrated that the calculator maintains predictive accuracy (age correlation 0.97, error = 3.6 years) across almost all body tissues including blood, brain, and bone.

Ambient Particle (Exposure) Assessment

We utilized the GEOS-chem chemical transport model (http://www.geos-chem.org; last accessed April 29, 2017) to generate 1-year exposure estimates for $PM_{2.5}$, sulfate, and ammonium. Sulfate and ammonium are the major $PM_{2.5}$ component species previously demonstrated to be most important in predicting DNAm-age (Nwanaji-Enwerem *et al.*, 2017). By incorporating meteorology variables, nonlinear chemistry, and detailed emissions inventories, GEOS-chem simulated the formation and transportation of atmospheric components and provided raw estimates of $PM_{2.5}$ and its major component species. Ten-fold cross-validation demonstrated that the model performed well for $PM_{2.5}$ mass and its component species with R^2s ranging from 0.70 to 0.88 (Di *et al.*, 2016). Each participant's residence was geocoded and linked to an area level grid-point. After accounting for address changes, we assigned particle estimates to each participant's address. Greater than 90% of NAS participants are retired; thus, home address exposures are expected to be a good proxy for their individual ambient exposures. We generated daily estimates at the 1×1 km area resolution and 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 participant's 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 (Nwanaji-Enwerem *et al.*, 2016).

Serum Endothelial Function Marker Assays

We used 3 common plasma endothelial function markers (vascular cell adhesion molecule-1 [VCAM], intercellular adhesion molecule-1 [ICAM], and vascular endothelial growth factor [VEGF]) that were measured in blood collected from NAS participants during their study visits. These markers were selected because of all serum physiologic markers available in the NAS, they are most specific and directly related to the endothelium. Other markers like CRP are nonspecific to the endothelium and have more nuanced relationships with other biological processes like general inflammation. These markers have also been consistently associated with $PM_{2.5}$ levels in numerous (NAS and nonNAS) epidemiologic studies and have been extensively used to assess endothelial function (Alexeeff *et al.*, 2011; Dai *et al.*, 2016). VCAM and ICAM are 2 important cellular adhesion molecules that mediate leukocyte-endothelial cell adhesion and transendothelial migration (Schnoor *et al.*, 2015). Laboratory staff measured VCAM (ng/ml) and ICAM (ng/ml) in serum using the enzyme-linked immunosorbent assay method (R&D Systems, Minneapolis, Minnesota). Sensitivity of the assay was 0.35 ng/ml for ICAM with day-to-day assay variabilities of 10.1, 7.4, 6.0, and 6.1% at concentrations of 64.2, 117, 290, and 453 ng/ml, respectively. Sensitivity of the assay was 2.0 ng/ml for VCAM with day-to-day assay variabilities of 10.2, 8.5 and 8.9% at concentrations of 9.8, 24.9, and 49.6 ng/ml, respectively. VEGF is a signaling protein that stimulates the production of endothelial cells and the formation of blood vessels (Jaipersad *et al.*, 2014). VEGF (pg/ml) was quantified using multiplexing technology (MILLIPLEXTM MAP) with commercially available MILLIPLEXTM MAP kits (EMD Millipore, Billerica, Massachusetts, USA). The VEGF assay has intra-assay and interassay precision of 13% and 19%, respectively.

Statistical Analysis

Primary analysis and variant scores. We first used linear mixed effects models to determine if we could observe previously published positive associations of 1-year $PM_{2.5}$, sulfate, and ammonium levels with DNAm-age in each of our 3 pathway subsets.

In our study reporting a relationship between $PM_{2.5}$ and DNAm-age, we used a tiered framework adjusting for confounders and covariates with: (1) *a priori* biological/clinical relevance and/or (2) reported in the existing literature (Nwanaji-Enwerem *et al.*, 2016). Tier 1 adjusted for chronological age and blood cell types. Tier 2 made additional adjustments for lifestyle and environmental factors. Tiers 3 and 4 expanded on tier 2 by additionally adjusting for age-related diseases and medications for age-related diseases, respectively. After examining model fit (assessed via AIC) and considering the implications of genetic polymorphisms on disease independent of $PM_{2.5}$ and DNAm-age relationships, we employed the tier 3 covariates for this analysis. In all, these models were adjusted for chronological age (continuous), 6 blood cell type estimates (ie, plasma cells, CD4+ lymphocytes, CD8+ lymphocytes, natural killer cells, monocytes, and granulocytes) (continuous) determined via Houseman and Horvath methods (Horvath, 2013; Houseman *et al.*, 2012), average 1-year temperature (continuous address-specific satellite measurements (Nwanaji-Enwerem *et al.*, 2017), 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), maximum years of education (continuous), 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 ≥140 mmHg or diastolic blood pressure ≥90 mmHg). The sulfate and ammonium models were additionally adjusted for $PM_{2.5}$ mass. All linear mixed effects models were run using the lme function from the nlme R package (Pinheiro *et al.*, 2014), and included a random participant-specific intercept to account for correlation between repeated outcome measures (ie, multiple visits for a participant).

We next used fully adjusted mixed effects models to determine: (1) if oxidative stress, endothelial function, or metal processing genetic variants were associated with DNAm-age and (2) the impact of air pollution-genetic pathway interactions on DNAm-age. To accomplish this, we utilized genotyping data from the NAS dataset and components of a novel genetic score

developed by Bind et al. (2014). Briefly, genotyping assays were performed using the Sequenom MassArray MALDI-TOF Mass Spectrometer with semi-automated primer design and implementation of the short extension method (San Diego, California). The MassArray system has the capacity to analyze multiple classes of genetic markers with high sensitivity.

Bind et al. (2014) developed a novel approach to investigate interactions between environmental exposures and the biological pathways of oxidative stress, endothelial function, and metal processing. The authors first related genes to 1 of these 3 pathways based on the biological functionality provided by GeneCards (Safran et al., 2010). Then considering independent outcomes representative of each pathway (8-hydroxy-2'-deoxyguanosine for oxidative stress, augmentation index for endothelial function, and patella lead concentration for metal processing), they used the least absolute shrinkage and selection operator (LASSO) method to select the most important gene variants for each of the outcomes.

Although the Bind et al. method would allow us to broadly identify pathways that may be related to the $PM_{2.5}$-DNAm-age relationship, it does not allow us to identify variants that are specifically important to this relationship. Identifying specific variants allows for a more comprehensive understanding of why these pathways are important. In an effort to identify pathway score component variants that were specifically sensitive to DNAm-age relationships, we made one alteration to the Bind et al. method. Two major limitations of LASSO selection are that (1) the number of selected variables are bounded by the number of observations and 2) that the LASSO tends to select 1 variable from a highly related group while ignoring the others (Lever et al., 2016). Given our desire to maximize the identification of specific genetic variants important to DNAm-age from 3 individual groups of pathway-related variants, the latter of these 2 limitations was a concern to us. To overcome this limitation, we employed an elastic net penalized regression, which allows for the selection of highly related variables (Zou and Hastie, 2005). Thus, starting with the reported Bind et al. list of candidate pathway-specific gene variants, we then employed an elastic net (penalized regression) via the glmnet function in the R glmnet package to determine which of these pathway-specific gene variants were also important for DNAm-age (Friedman et al., 2010). Our method was similar to that described by Lenters et al. (2016) and the full documentation for running all aspects of the elastic net via glmnet is publically available (https://cran.r-project.org/web/packages/glmnet/index.html; last accessed April 29, 2017). In short, all aforementioned covariates were included in the elastic net regression models as unpenalized variables. The fully-adjusted elastic net regression linear models utilized a hybrid of ridge and LASSO penalty functions to determine which genetic variants, within each respective pathway, were important predictors of DNAm-age. With ridge, the square of the regression coefficients for predictors are penalized. All predictors are retained but coefficients from highly related predictors are proportionally shrunk towards zero. With LASSO, the absolute value of predictor coefficients is penalized and coefficients are shrunk by a constant factor. Coefficients for the least predictive variants are shrunk to zero and only one predictor from a highly correlated group tends to be selected. By combining both of these penalty functions, the elastic net performed selection while allowing for the inclusion of highly-related genetic variants bin (Friedman et al., 2010; Zou and Hastie, 2005). Cross-validation was also performed to determine the optimal degree of penalization. The proportion of ridge and LASSO functions and the corresponding penalty that yielded the minimum mean-squared error of prediction from repeated 10-fold cross-validation was used in the final elastic net selection model. Gene variants with nonzero coefficients are considered as "selected" by the elastic net. Following this elastic net selection, we were left with 3 lists: (1) Oxidative stress gene variants that were important for DNAm-age; (2) Endothelial function gene variants that were important for DNAm-age; and (3) Metal processing gene variants that were important for DNAm-age.

Returning to the original Bind et al. methodology, we then summed the sign of the nonzero coefficients for each of the important variants to construct pathway specific variant scores for all study participants. For instance, say hypothetical oxidative stress variants A1, A2, and B3 had elastic net coefficients of $+2.3$, -1.7, and 1.6. A participant with all of these variants would have an oxidative stress polymorphism score of $+1$ (ie, $1 - 1 + 1 = 1$). Another participant with only variants A1 and B3 would have an oxidative stress score of $+2$ (ie, $1 + 1 = 2$). Final binary pathway polymorphism scores were created by dichotomizing each score as low or high aging-risk using the median of each score's distribution in the study sample.

Again, we used fully adjusted mixed effects models to determine if each pathway polymorphism score was independently associated with DNAm-age. We then included interaction terms of our main exposures ($PM_{2.5}$, sulfate, and ammonium) with the 3 respective pathway polymorphism scores to identify genetic pathway-air pollution interactions that are related to DNAm-age.

Secondary analysis. Although our air pollution-genetic pathway interaction models provided us with some insight to biological pathways that may be genetically relevant to DNAm-age, we further investigated if these same pathways had functional relationships with DNAm-age. Thus, we first looked to see if serum endothelial function markers were correlated with DNAm-age in our study sample. We next constructed linear mixed effects models to see if the serum markers were significantly associated with DNAm-age after adjusting for chronological age, blood cell types, and the age-related diseases of lifetime cancer diagnosis, hypertension, diabetes, and coronary heart disease.

All statistical analyses were performed using R Version 3.1.1 (R Core Team, Vienna, Austria) and we considered a P-value $< .05$ to be statistically significant.

RESULTS

Descriptive Statistics

The demographics and clinical data of all study participants in each of the 3 biological pathway subsets are presented in Table 1. Participants in each subset had an average age (SD) and an average DNAm-age (SD) both of approximately 74 ± 7 years. Supplementary Table 1 lists all of the candidate pathway-specific genetic variants used by Bind et al. (2014). Supplementary Table 2 lists the genetic variants that were selected by the elastic net as important for DNAm-age. No metal processing variants were selected by the elastic net; thus, no variant score could be calculated for that subset. Participants in the oxidative stress subset had a variant scores ranging from -6 to 4, and endothelial function subset participants had variant scores ranging from -3 to 0. Both subsets had variant scores with a median of 1.

Table 2 summarizes the mean levels of $PM_{2.5}$, sulfate, and ammonium in each of the 3 subsets. In the oxidative stress subset, 10.3 (2.14), 3.40 (0.80), and 1.04 (0.29) $\mu g/m^3$ were the average

Table 1. Characteristics of Study Subjects, 2000–2011

Variable	Oxidative Stress Subset (n = 702)[a]	Endothelial Function Subset (n = 779)[a]	Metal Processing Subset (n = 744)[a]
Age, mean (SD)	74.7 (6.89)	74.8 (6.95)	74.7 (6.80)
DNAm-age, mean (SD)	74.2 (7.97)	73.9 (7.92)	73.8 (7.51)
Variant score, mean (range)	−0.51 (−6, 4)	−1.23 (−3, 0)	—
Temperature, mean (SD)	11.3 (0.99)	11.3 (0.98)	11.3 (1.03)
Pack years, mean (SD)	20.4 (24.7)	20.6 (24.7)	20.9 (24.9)
Smoking status, n (%)			
Current	35 (5)	37 (5)	34 (5)
Former	450 (64)	512 (65)	480 (65)
Never	217 (31)	230 (30)	230 (30)
Season, n (%)			
Spring	177 (25)	192 (25)	188 (25)
Summer	146 (21)	164 (21)	157 (21)
Fall	238 (34)	267 (34)	251 (34)
Winter	141 (20)	156 (20)	148 (20)
BMI, n (%)			
Healthy/Lean	168 (24)	189 (24)	172 (23)
Overweight	370 (53)	406 (52)	391 (53)
Obese	164 (23)	184 (24)	181 (24)
Alcohol consumption, n (%)			
<2 drinks/day	560 (80)	620 (80)	599 (81)
≥2 drinks/day	142 (20)	159 (20)	145 (19)
Education, n (%)			
≤12 years	192 (27)	207 (27)	192 (26)
12–16 years	320 (46)	355 (46)	341 (46)
>16 years	190 (27)	217 (27)	211 (28)
Lifetime cancer diagnosis, n (%)			
Yes	390 (55)	435 (56)	426 (57)
No	312 (45)	344 (44)	318 (43)
Coronary heart disease, n (%)			
Yes	221 (31)	261 (34)	257 (35)
No	481 (69)	518 (66)	487 (65)
Diabetes, n (%)			
Yes	120 (17)	138 (18)	131 (18)
No	582 (83)	641 (82)	613 (82)
Hypertension, n (%)			
Yes	514 (73)	571 (73)	541 (73)
No	188 (27)	208 (27)	203 (27)

[a]From 552 participants (940 visits), we excluded participants missing pathway-specific variant data. This resulted in 3 distinct, but not mutually exclusive, subsets.

Table 2. Mean 1-Year Particulate Matter 2.5 ($PM_{2.5}$), Sulfate, and Ammonium Concentrations, 2000–2011

Particle (μg/m^3)	Oxidative Stress Subset (n = 702)	Endothelial Function Subset (n = 779)	Metal Processing Subset (n = 744)
$PM_{2.5}$, mean (IQR)	10.3 (2.14)	10.3 (2.09)	10.3 (2.09)
Sulfate, mean (IQR)	3.40 (0.80)	3.38 (0.80)	3.42 (0.84)
Ammonium, mean (IQR)	1.04 (0.29)	1.04 (0.29)	1.04 (0.28)

(IQR) levels of $PM_{2.5}$, sulfate, and ammonium, respectively. Both the endothelial function and metal processing subsets showed similar levels of these particles. The mean (IQR) levels for $PM_{2.5}$, sulfate, and ammonium in the endothelial function subset were 10.3 (2.09), 3.38 (0.80), and 1.04 (0.29) μg/m^3. The mean (IQR) levels for same particles in the metal processing subset were 10.3 (2.09), 3.42 (0.84), and 1.04 (0.28) μg/m^3.

1-Year Particle Levels and Variant Scores as Predictors of DNAm-Age

Table 3 summarizes the results of linear mixed effects models where dichotomized variant scores and IQR increases in 1-year particle levels were modeled as independent predictors of DNAm-age. In the endothelial function subset, an IQR increase in 1-year $PM_{2.5}$ ($\beta = 0.67$, $P = .005$), sulfate ($\beta = 0.64$, $P < .0001$), and ammonium ($\beta = 0.49$, $P = .002$) were all significantly, positively associated with DNAm-age. 1-year IQR increases in all 3 particles were also significant positive predictors of DNAm-age in the metal processing subset. Similar trends were observed in the oxidative stress subset where sulfate ($\beta = 0.64$, $P < .0001$) and ammonium ($\beta = 0.58$, $P = .0005$) were significant positive predictors of DNAm-age and $PM_{2.5}$ ($\beta = 0.46$, $P = .07$) was a marginally significant positive predictor of DNAm-age.

A total of 13 oxidative stress variants and 3 endothelial function variants were selected by the elastic net as important for

predicting DNAm-age. These variants were used to calculate the variant scores for these pathways (Supplementary Table 2). Again, no variants were selected by the elastic net for the metal processing subset; hence, no variant score could be calculated for this biological pathway. In both the oxidative stress and endothelial function subsets, individuals with high aging-risk variant scores (>median) on average had at least a 0.62-year higher DNAm-age than their counterparts with low aging-risk variant scores. However, these relationships were not statistically significant (Table 3).

Effect Modification by Variant Scores

Figures 1 and 2 depict the modifying role of the oxidative stress (Figure 1) and endothelial function (Figure 2) variant scores on the relationship of 1-year particle exposures with DNAm-age. The association of all 3 particles with DNAm-age was greater in individuals with a low aging-risk oxidative stress variant score when compared with individuals with a high aging-risk oxidative stress variant score (Figure 1), but none of these differences were statistically significant. The effect of all 3 particles on DNAm-age was greater in individuals with a high aging-risk endothelial function variant score when compared with individuals with a low aging-risk endothelial function variant score (Figure 2). These differences were significant for sulfate and ammonium exposure but not quite for $PM_{2.5}$. The relationships in Figure 1 are quantified in Supplementary Table 3.

Relationships of Serum Endothelial Function Markers With DNAm-Age

In our secondary analysis, DNAm-age was significantly positively correlated with both ICAM ($r = 0.13$, $P = .0001$) and VCAM ($r = .25$, $P < 0.0001$) (Supplementary Table 4). However, DNAm-age was not significantly correlated with VEGF ($r = 0.02$, $P = .54$). After adjusting for chronological age, blood cell types, cancer, hypertension, diabetes, and coronary heart disease, ICAM ($\beta = 0.01$, $P = .005$) and VCAM ($\beta = 0.002$, $P = .004$) were both significant positive predictors of DNAm-age. VEGF ($\beta = -0.00003$, $P = .82$) was not significantly associated with DNAm-age (Table 4).

Endothelial Function Variant Score as a Modifier of the Association of PM2.5 With ICAM and VCAM

We performed subsequent analyses, using fully-adjusted mixed effects models, where we found that the positive associations of 1-year particle levels with ICAM (Supplementary Figure 1) and VCAM (Supplementary Figure 2) were greater in individuals with high aging-risk endothelial function variant scores when compared with their counterparts. These findings were only statistically significant for ICAM.

Table 3. Mean 1-Year Particulate Concentrations and Variant Score as Independent Predictors of DNAm-Age

Predictor	Oxidative Stress Subset (n = 702)		Endothelial Function Subset (n = 779)		Metal Processing Subset (n = 744)	
	Difference in DNAm-age (years) for IQR (95% CI)	*P*	Difference in DNAm-age (years) for IQR (95% CI)	*P*	Difference in DNAm-age (years) for IQR (95% CI)	*P*
$PM_{2.5}$	0.46 (−0.04, 0.97)	.07	0.67 (0.21, 1.15)	**.005**	0.48 (0.003, 0.94)	**.05**
Sulfate	0.64 (0.38, 0.89)	**<.0001**	0.64 (0.40, 0.88)	**<.0001**	0.53 (0.29, 0.77)	**<.0001**
Ammonium	0.58 (0.25, 0.91)	**.0005**	0.49 (0.18, 0.80)	**.002**	0.53 (0.16, 0.89)	**.005**
Variant score[a]						
Low	ref	—	ref	—	—	—
High	0.62 (-0.65, 1.89)	.34	0.67 (−0.46, 1.79)	.25	—	—

All models adjusted for chronological age, blood cell type, temperature, pack years, smoking status, season, BMI, alcohol consumption, education, lifetime cancer diagnosis, hypertension, diabetes, and coronary heart disease. Sulfate, ammonium and variant score models are additionally adjusted for total $PM_{2.5}$ mass.
[a]Binary pathway variant scores were created by dichotomizing each score using the median of each score's distribution in the study sample. Participants had a low aging-risk if their score was less than (<) the median and a high aging-risk score if their score was greater than or equal to (≥) the median.

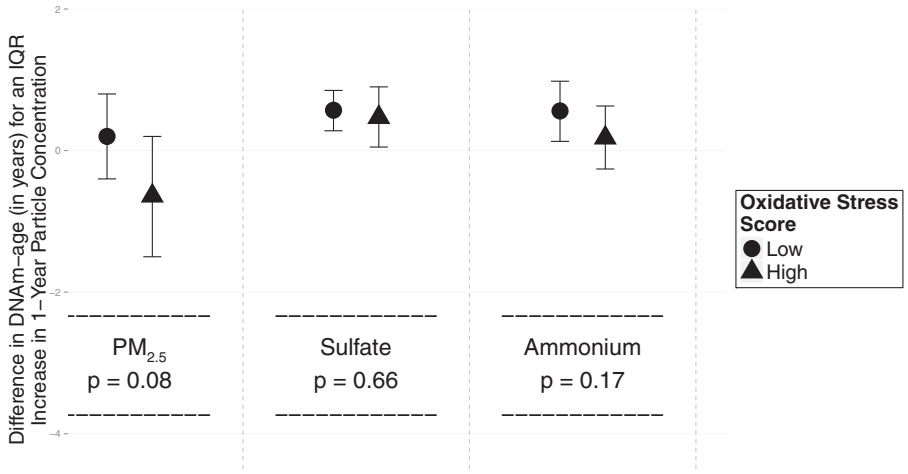

Figure 1. Difference in DNAm-age for one interquartile range increase in 1-year particle exposure according to oxidative stress score (low vs high) in the fully adjusted linear mixed effects model.

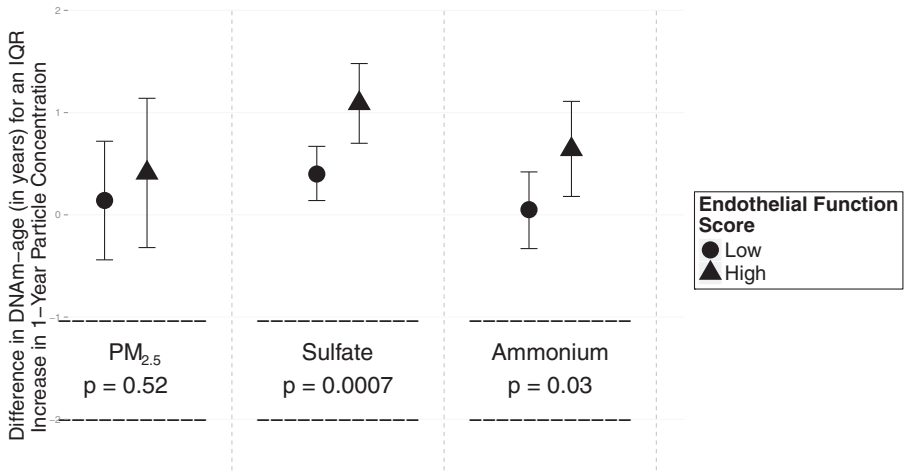

Figure 2. Difference in DNAm-age for one interquartile range increase in 1-year particle exposure according to endothelial function score (low vs high) in the fully adjusted linear mixed effects model.

Table 4. Associations of Serum Endothelial Function Markers With DNAm-Age

Marker	Difference in DNAm-age (95% CI)	P	n
ICAM (ng/ml)	0.01 (0.002, 0.012)	**.005**	608
VCAM (ng/ml)	0.002 (0.0005, 0.0026)	**.004**	608
VEGF (pg/ml)	−0.00003 (−0.0003, 0.0003)	**.82**	608

Models adjusted for chronological age, blood cell type, lifetime cancer diagnosis, hypertension, diabetes, and coronary heart disease.

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.

DISCUSSION

This study employed a large longitudinal cohort of elderly men to: (1) identify pathway-specific genetic variants that were related to DNAm-age and (2) determine if these variants modified the association of $PM_{2.5}$ and $PM_{2.5}$ component levels with DNAm-age. In each of our pathway subsets, we first wanted to ensure that we observed similar relationships of $PM_{2.5}$, sulfate, and ammonium with DNAm-age as previously reported. Indeed, this was the case as sulfate and ammonium were significant positive predictors of DNAm-age in all 3 subsets. $PM_{2.5}$ levels were significant positive predictors of DNAm-age in the endothelial function and metal processing subsets, while being marginally significant in the oxidative stress subset—potentially due to reduced power attributed to the subset's slightly reduced sample size.

We next used components of a method developed by Bind *et al.* (2014) to calculate variant scores for oxidative stress, endothelial function, and metal processing pathways. The addition of an elastic net selection to the Bind *et al.* method allowed us to optimize the sensitivity of the approach to DNAm-age relationships, while allowing for the identification of genetic-pathway variants that were specifically important for DNAm-age. Given that each of these pathways is known to be associated with $PM_{2.5}$-related disease, we predicted that the elastic net would select important variants from each of the pathways. This was the case for oxidative stress and endothelial function, but not metal processing. Published literature has already demonstrated that these metal processing variants do not modify the effect of $PM_{2.5}$ levels on a panel serum physiological markers including fibrinogen, ICAM, and CRP (Bind *et al.*, 2014). Thus, it is possible that metal processing pathways have little or no relationship with DNAm-age physiology especially in the context of $PM_{2.5}$ exposure. Nevertheless, no studies have examined the relationship of $PM_{2.5}$'s metal component species with DNAm-age and no studies have examined the relationship of direct metal exposures with DNAm-age. Such studies will be necessary to confirm our null findings of metal processing physiology with DNAm-age.

A total of 13 oxidative stress variants and 3 endothelial function variants were selected by the elastic net. A subsequent literature review revealed that many of the selected oxidative stress variants, including rs2284367 (CAT), rs2300181 (CAT), and rs1799811 (GSTP1), have already been implicated as effect modifiers of the relationship of $PM_{2.5}$ and its component species with numerous health endpoints (Ren *et al.*, 2010a,b). None of the selected endothelial function variants had been implicated in $PM_{2.5}$ relationships, but there is evidence of their role in impacting disease susceptibility following other environmental insults like radiation, cigarette smoke, and pesticide exposures (Ayala-Haedo *et al.*, 2010; Hancock *et al.*, 2008; Zhang *et al.*, 2014). After using the direction of the elastic net coefficients of these variants to construct pathway-specific variant scores, we determined if these scores were associated with DNAm-age. Given that the Bind *et al.* method constructs the scores such that a higher score correlates with a profile of higher risk for increased DNAm-age, we expected that individuals with high oxidative stress or endothelial function scores would on average have higher DNAm-ages than their low score counterparts. After examining the effect estimates, this was the case. High aging-risk oxidative stress score participants on average had a 0.62-year greater DNAm-age and high aging-risk endothelial function participants on average had a 0.67-year greater DNAm-age. Despite these trends, these results were not statistically significant.

When we next explored the modifying role of these variant scores on particle-DNAm-age relationships, we found that the

effect of sulfate and ammonium on DNAm-age were on average approximately 0.60-years greater in participants with a high aging-risk endothelial function score when compared with participants with a low aging-risk endothelial function score. A similar trend was observed with $PM_{2.5}$ and endothelial score interactions, but this was not statistically significant. This result suggests that DNAm-age is sensitive to endothelial function physiology and is further supported by our secondary analysis that revealed significant associations of ICAM and VCAM with DNAm-age after adjusting for covariates. Numerous human and animal studies have demonstrated that $PM_{2.5}$ exposure upregulates expression of endothelial factors, which are known to play a role in vascular dysfunction (Wu *et al.*, 2016; Xiao *et al.*, 2016). Moreover, vascular physiology is a ubiquitous component of many disease processes and may help explain why DNAm-age has been linked to all-cause mortality, malignancy, cognitive deficits, and a host of other diseases (Mendonca *et al.*, 2016; Toth *et al.*, 2016). Endothelial micro-particles from acute coronary artery patients (a surrogate marker of endothelial dysfunction) have been shown to promote thrombogenecity and aging phenotypes in healthy coronary artery cells (Abbas *et al.*, 2016). In a cross-sectional study, endothelial VCAM was associated with increased vascular resistance and lower cognitive performance (Tchalla *et al.*, 2016). On the contrary, pharmaceutical agents that are used to treat age-related disease (eg, statins) have been shown to increase endothelial progenitor cells, which may promote endothelial repair and offer benefits like cardioprotection (Ricottini *et al.*, 2016).

It is interesting that endothelial variants significantly modified the associations of sulfate and ammonium, but not total $PM_{2.5}$. As it is widely accepted that different $PM_{2.5}$ species have different toxicological effects, this finding may speak to a specific toxicity of these component species via endothelial function pathways (Zanobetti *et al.*, 2009). It has already been demonstrated that ammonium and sulfate moieties can impact endothelial function (Ando and Fujita, 1994; Lin *et al.*, 2016). It is also important to highlight that VEGF was not significantly correlated with DNAm-age. VEGF was also not significantly associated with DNAm-age after adjusting for covariates. The differences between DNAm-age's relationship with VEGF compared with its relationship with ICAM or VCAM could possibly be attributed to VEGF gene and protein expression. VEGF production is induced in cells that are hypoxic and circulating VEGF then binds to endothelial cells to promote angiogenesis (Jaipersad *et al.*, 2014). ICAM and VCAM are more specifically produced by endothelial cells and play a prominent role in endothelial cell interactions with inflammatory cells (Schnoor *et al.*, 2015). Hence, our data allude to a specific relationship between DNAm-age and endothelial function that may be related to immune regulation. This finding is particularly promising, as the immune system has long been thought to play a role in the adverse effects of $PM_{2.5}$ (Zhong *et al.*, 2015). In all, our findings and the existing literature suggest that the endothelial function pathway is a promising place to begin understanding the molecular relevance of DNAm-age. Future studies including these and other endothelial function markers are necessary to confirm our findings and further define this relationship.

Finally, none of the particle-oxidative stress score interactions were statistically significant, but it is worth noting the direction of the effect estimates for these interaction terms. Like the endothelial function score, individuals with a high aging-risk oxidative stress score had a higher DNAm-age when compared with individuals with a low aging-risk oxidative stress scores (results not statistically significant). However, unlike the endothelial function score, a high aging-risk oxidative stress score appeared to mitigate the effect of particles of DNAm-age. These results were not statistically significant but may suggest competing effects between high particle exposure and a high aging-risk oxidative stress physiology. When both are simultaneously present (ie, the interaction of both variables is considered) they appear to inhibit or dampen each other's effects. Such a phenomenon is often observed in epidemiologic research and biological systems (Fan *et al.*, 2015). One air pollution related example of competing effects is the mitigation of associations of particles with the birth complication preeclampsia when multiple particle sources are considered simultaneously (Dadvand *et al.*, 2014).

The strengths of this study include utilization of novel biomarker and genetic pathway tools, rigorous statistical methods, and a large longitudinal cohort with repeated measures of ambient pollutant exposures, DNA methylation, and potential confounders. This is the first study to use genetic variants to study the relationship of ambient particles with DNAm-age. On the contrary, our study does have some notable limitations. First, given that a majority of NAS participants are retired and are very likely to spend most of their time at home, we use a validated chemical transport model to estimate 1-year ambient levels of $PM_{2.5}$, sulfate, and ammonium at participants' addresses. Such an approach at estimating personal exposures could potentially result in nondifferential misclassification, but this is likely to attenuate statistical associations rather than bias them away from the null (Kioumourtzoglou *et al.*, 2014; Weisskopf and Webster, 2017). Second, we employ a genetic variant score approach that is somewhat limited because it does not provide genome-wide resolution of the 3 biological pathways. Nonetheless, the variants that are present are representative of their respective pathways. Third, to maximize statistical power, we used our full cohort to calculate variant scores and test for effect modification of DNAm-age relationships. This could be a source of bias and is a limitation of this study. Still, our subsequent analysis demonstrating that the endothelial function variant score was a significant modifier of the $PM_{2.5}$-ICAM relationship demonstrates that the variation captured by our score impacts the association of $PM_{2.5}$ with endothelial function markers that are independent of DNAm-age. Notwithstanding this evidence, validation of our score and findings in an independent cohort is a future direction of this work. Fourth, all bisulfite-mediated methods used for quantifying DNA methylation are limited in their ability to distinguish between 5-methylcytosine and its oxidation product 5-hydroxymethylcytosine (Reinders and Paszkowski, 2010). Last, our findings are based on an elderly cohort of Caucasian males that reside in a lightly polluted environment. To date, only one study has explicitly examined race and sex differences in DNAm-age and data from that study suggests that men have higher DNAm-ages than women (Horvath *et al.*, 2016). Furthermore, there is evidence that race and sex differences can impact individual responses to $PM_{2.5}$ exposure. For instance, one study reported that urban $PM_{2.5}$ levels were associated with asthma exacerbations in African Americans, but not Caucasian Americans (Glad *et al.*, 2012). Nevertheless, more work must be done to confirm if these, and similar, reported differential effects are truly due to race/sex or if they are instead due to differences in residential characteristics and other social determinants. A limited amount of research has explicitly explored the race or sex differences of the endothelial function variants selected by our elastic net. One study examining gene-gene interactions that influence pulmonary tuberculosis susceptibility reported strong interactions between

the rs2248814 (NOS2A) variant and other genes in African Americans but not Caucasians (Velez *et al.*, 2009). Another study reported that the rs1800779 (NOS3) variant was positively associated with high tension primary open glaucoma in women, but not in men (Kang *et al.*, 2010). In regards to the results of this study, additional studies involving other demographic groups, in different environments, and using other assessments of endothelial function will be necessary to confirm our findings more broadly.

In summary, our findings add evidence that genetic variation can impact the association of long-term fine particle levels with DNAm-age. In particular, the effect of 1-year particle levels on DNAm-age was greater in individuals with a high aging-risk endothelial function genetic variant profile when compared with individuals with a low aging-risk variant profile. We also report novel, robust positive associations of serum endothelial markers with DNAm-age. Although the biological relevance of DNAm-age is still greatly undefined, our study makes a valiant, early attempt at addressing this important research gap. Again, future studies in different populations using these and other endothelial markers will be necessary to broaden the understanding of the relationship of endothelial function with DNAm-age.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

ETHICS APPROVAL

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

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