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Impacts of the Mitochondrial Genome on the Relationship of Long-term Ambient Fine Particle Exposure with Blood DNA Methylation Age

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Abstract

The mitochondrial genome has long been implicated in age-related disease, but no studies have examined its role in the relationship of long-term fine particle (PM2.5) exposure and DNA methylation age (DNAm-age) – a novel measure of biological age. In this analysis based on 940 observations between 2000 and 2011 from 552 Normative Aging Study participants, we determined the roles of mitochondrial DNA haplogroup variation and mitochondrial genome

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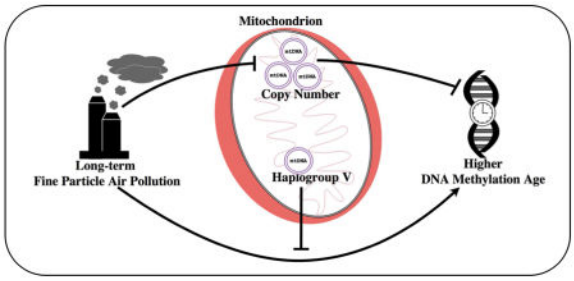
abundance in the relationship of PM2.5 with DNAm-age. We used the GEOS-chem transport model to estimate address-specific, one-year PM2.5 levels for each participant. DNAm-age and mitochondrial DNA markers were measured from participant blood samples. Nine haplogroups (H, I, J, K, T, U, V, W, and X) were present in the population. In fully-adjusted linear mixed-effects models, the association of PM2.5 with DNAm-age (in years) was significantly diminished in carriers of haplogroup V (*Pinteraction*= 0.01; β= 0.18, 95%CI: −0.41, 0.78) compared to non-carriers (β= 1.25, 95%CI: 0.58, 1.93). Mediation analysis estimated that decreases in mitochondrial DNA copy number, a measure of mitochondrial genome abundance, mediated 12% of the association of PM2.5 with DNAm-age. Our data suggests that the mitochondrial genome plays a role in DNAm-age relationships particularly in the context of long-term PM2.5 exposure.

Graphical Abstract



Keywords

DNA methylation age; Particulate matter 2.5; Mitochondrial genome

1 Introduction

Research continues to implicate long-term fine particulate air pollution (PM2.5) as a major risk factor for aging and age-related disease. For instance, a recent study of over 500 elderly individuals reported a 27% increase in the risk of an individual developing metabolic syndrome for every 1 μg/m^3 increase in annual PM2.5 concentration[1]. In addition to metabolic disease, long-term PM2.5 exposure has been associated with an increased likelihood of all-cause mortality[2] and significant deficits in cardiac autonomic function[3], cognitive performance[4], and respiratory ability[5]. Even on a molecular level, researchers have consistently demonstrated relationships of PM2.5 with more traditional measures of biological aging including, telomere length[6] and inflammatory markers[7]. These PM2.5-biological aging relationships are of particular interest because they often persist independent of age-related diseases and they may offer insight as to how PM2.5 interacts with biological systems to adversely impact human health. Recently, researchers reported positive associations of long-term PM2.5 with DNA methylation age (DNAm-age)[8], a novel tissue-independent measure of biological age calculated from DNA methylation values at 353 age-correlated CpG dinucleotides[9]. Furthermore, the authors examined the relationships of five major PM2.5 component species (ammonium, elemental carbon, organic carbon, nitrate, and sulfate) with DNAm-age, and found that sulfate and ammonium were most associated with DNAm-age[10]. Although the ability of DNAm-age to reflect previous

environmental exposures and predict multiple health outcomes makes it a promising biomarker of aging[11–15], it is so novel that the molecular implications of these relationships remain largely unknown. Addressing this research gap is of paramount importance for future aging research involving this biomarker.

Mitochondria are membrane-bound intracellular organelles tasked with energy production and highly involved in biological aging processes[16–17]. Mitochondria possess their own genomes which exist as circular double-stranded molecules of DNA that code for a number of biological effectors including some major components of the energy-generating electron transport chain (ETC)[18–19]. Due to the mitochondrial genome's proximity to the ETC (the major source of intracellular reactive oxygen species) and its diminished DNA repair capacity (in comparison to nuclear DNA), mitochondrial DNA is particularly susceptible to oxidative damage[20]. There is also convincing evidence that the mitochondrial genome is susceptible to damage from exogenous oxidative stressors[21–23]. Overall, it is the damage to the mitochondrial genome that has been specifically linked to accelerated aging[24–25]. Given that PM2.5 exposure is a known risk factor of systemic oxidative stress[26] and since PM2.5 exposure has already been linked to mitochondrial genome integrity[27–28], we hypothesized that the mitochondrial genome may be involved in the PM2.5-DNAm-age relationship.

In the present study, we examined the impact of mitochondrial genomic variation and abundance (a compensatory response to poor mitochondrial genome integrity) on the relationship of PM2.5 and its component species with DNAm-age. First, we examined if different mitochondrial haplogroups (forms of normal mitochondrial genetic variation that potentially impact ETC capacity)[29–30] modified the association of PM2.5 and its component species with DNAm-age. Next, we determined the relationship of one commonly used measure of mitochondrial genome abundance, mitochondrial DNA copy number, with DNAm-age. Mitochondrial copy number is the ratio of a cell's mitochondrial DNA to nuclear DNA. Fluctuations in mitochondrial copy number often occur with normal mitochondrial biogenesis and degradation, but the measure is also sensitive to exogenous stressors and is thought to be an adaptive response to compensate for mitochondrial genome damage[31]. Copy number has already been associated with PM2.5 levels[32], but no studies have examined its relationship with DNAm-age. Finally, we determined if copy number mediated and/or modified the association of PM2.5 with DNAm-age.

2 Materials and Methods

2.1 Study Population

Participants in the present analysis were active participants in the Veteran Affairs Normative Aging Study (NAS), a longitudinal cohort study of aging established in 1963[33]. The NAS is a closed cohort of now elderly community-dwelling men living in the Greater Boston area. At enrollment, all participants were free of chronic diseases. Participants return every 3 to 5 years for onsite, follow-up study visits. During these recurring visits, participants receive comprehensive outpatient medical evaluations, bio-specimens (including blood) are collected, and participants provide detailed information about their diets and other lifestyle factors that may affect their health. All participants provided written informed consent to the

VA Institutional Review Board (IRB), and human subjects approval was granted by the VA and Harvard T.H. Chan School of Public Health IRBs (protocol 14027-102).

All NAS men with continued study participation as of the year 2000, when address-specific PM2.5 component species levels became available, were eligible for the present study sample. We began with a total of 552 participants with 940 observations between the years 2000 and 2011. This was the study sample that was used in reporting the significant associations between PM2.5 component species and DNAm-age in our previous publication[10]. Of these 552 participants, 249 (45%) had one visit, 218 (40%) had two visits, and 85 (15%) had three or more visits. From this sample, we then excluded participants missing mitochondrial haplogroup data. This resulted in a final study sample of 508 participants with 870 total study visits. In the final study sample, 227 participants (45%) had one visit, 200 (39%) had two visits, and 81 (16%) had three or more visits.

2.2 DNA Methylation and DNA Methylation Age (DNAm-age)

Whole blood was collected from each participant during each NAS follow-up visit. We performed bisulfite conversion (EZ-96 DNA Methylation Kit, Zymo Research, Orange, CA, USA) on extracted DNA from the buffy coat of the whole blood, and then used the Illumina Infinium HumanMethylation450 BeadChip to measure the DNA methylation of CpG probes. To minimize batch effects and ensure a similar age distribution across chips and plates, we randomized chips across plates and used a two-stage age-stratified algorithm to randomize samples. For quality control, we removed samples where >5% of probes had a beadcount < 3 or > 1% of probes had a detection P-value >0.05. After pre-processing the remaining samples with Illumina-type background correction without normalization and normalizing the samples with dye-bias and BMIQ3 adjustments, we generated methylation beta values[34]. Beta values represent the percentage of methylation for each of the ~480,000 CpG sites in the BeadChip array. In other words, beta = intensity of the methylated signal (M)/[intensity of the unmethylated signal (U) + intensity of the methylated signal (M) + 100].

DNAm age was calculated using Horvath's publically available online calculator (http://labs.genetics.ucla.edu/horvath/dnamage/). DNAm-age was derived from an elastic net penalized regression run on multiple data sets of different cell and tissue types. After 21,369 CpG probes – shared by both 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)[9]. The model coefficients from these 353 CpGs were used by the calculator to predict the age of each DNA sample (*i.e.* DNAm-age). The calculator maintains predictive accuracy (age correlation 0.97, error = 3.6 years) across almost all body tissues including blood and brain[9].

2.3 Fine Particulate (PM2.5) Air Pollution

We used the simulation outputs from GEOS-chem, a chemical transport model[35], fused with land-use variables to generate one-year exposure estimates for PM2.5 as well as sulfate and ammonium, the major PM2.5 component species demonstrated to be most important in predicting DNAm-age[10]. Ten-fold cross-validation demonstrated that the model performed well for PM2.5 mass and its component species with R^2s ranging from 0.70 to 0.88[36].

Existing literature demonstrates that the one-year PM2.5 exposure window is robustly associated with DNAm-age[8]. We generated daily estimates at the 1 km ×1 km area resolution and one-year total PM2.5 and PM2.5 component species exposure windows by averaging daily exposures for the 365 days prior to the day of each participants' NAS visit. Given that greater than 90% of NAS participants are retired, home address exposures are expected to be a good proxy for their individual ambient exposures. After geocoding and linking participants' residencies to an area level grid-point; and accounting for address changes and time spent away from home (>7 days), we assigned particle estimates to each participant's address.

2.4 Mitochondrial (DNA) Haplogroups

Participant blood was genotyped using Taqman or Sequenom assays (Applied Biosystems, Foster City, CA)[37]. All samples were successfully genotyped and internal blinded quality control samples were >99% concordant. Hardy-Weinberg equilibrium tests were not assessed because they are not valid for mitochondrial polymoprhisms[38]. No heteroplasmy (heterozygous samples) were observed. Nine mitochondrial DNA haplogroups (H, I, J, K, T, U, V, W, and X) were observed in the cohort and all are common to populations of European ancestry. Based on the phylogenetic evolutionary tree and restriction fragment length polymorphisms, these haplogroups can be grouped into four clusters (Cluster 1:J,T; Cluster 2: V,H; Cluster 3: U,K; Cluster 4: I, W, X)[39]. The clusters are widely known, and since overall type I error increases as the number of statistical tests increases, many epidemiologic studies first perform cluster analyses[37, 40]. Following this framework, we perform primary cluster analyses and subsequently explore individual haplogroups of interest.

2.5 Mitochondrial DNA Copy Number (Genome Abundance)

As noted, the mitochondrial genome is particularly vulnerable to both endogenous and exogenous (*e.g.* air pollution) oxidative stressors due to its proximity to the ETC, lack of protective barriers (*i.e.* histone proteins, chromatin organization, etc.), and relatively limited DNA damage repair activity[41]. We are utilizing one measurement of mitochondrial genome abundance (copy number) that is sensitive to oxidative stress. Mitochondrial copy number represents the ratio of mitochondrial DNA copy number to the nuclear DNA copy number (mtDNA:nDNA) and was also calculated from whole blood samples collected at every visit. As previously described[31], real-time PCR (RT-PCR) is used to measure the ratio of a mitochondrial gene (mtDNA 12S ribosomal ribonucleic acid) to a nuclear gene (Ribonuclease P gene), which is normalized to a reference DNA sample (a pool of 300 test samples) to obtain relative mitochondrial DNA copy number values controlled for plate effects.

2.6 Statistical Analysis

Covariates—The relationships of the mitochondrial genome with DNAm-age and its role in the association of PM2.5 with DNAm-age were evaluated using linear mixed-effects models including a random participant-specific intercept to account for correlation between repeated outcome measures (*i.e.* multiple visits for a participant). In the analyses, we controlled for the following covariates *a priori* based on previous analyses[8, 10] and the

relevant literature[42–43]: chronological age (continuous), blood cell proportions [plasma cells, CD4+ lymphocytes, CD8+ lymphocytes, natural killer (NK) cells, monocytes, and granulocytes] (continuous) determined via Houseman and Horvath methods[9, 44], average 1-year temperature (continuous address-specific satellite measurements[10]), 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).

Direct Associations—We first used fully-adjusted linear mixed effects models to evaluate previously published positive associations of one-year PM2.5, sulfate, and ammonium levels with DNAm-age. Sulfate and ammonium models were additionally adjusted for PM2.5 mass. To limit multiple comparisons and the potential for false positive results, we performed mitochondrial haplogroup cluster analyses – as conducted in a previously published NAS study of haplogroups[37] – evaluating the direct relationships of mitochondrial haplogroup clusters with DNAm-age and mitochondrial DNA copy number. We also used fully-adjusted mixed-effects models to determine the associations of mitochondrial DNA copy number with DNAm-age.

Mitochondrial Haplogroup as an Effect Modifier—Since haplogroup is a genetic parameter that does not change during life, it is not on the causal pathway of the exposure and outcome but could potentially impact the relationship of the exposure with the outcome. For these reasons, it appropriate to consider it as an effect modifier. Specifically, we evaluated if the haplogroup clusters modified the associations of PM2.5, sulfate, and ammonium with DNAm-age. In these analyses the reference group were all participants without the cluster of interest. For example, when we evaluated the modifying role of mitochondrial haplogroup cluster 1 on the association of PM2.5 with DNAm-age, we compared participants genotyped as having cluster 1 against all other participants (*i.e.* participants genotyped as having clusters 2, 3, and 4). Structuring the analyses this way allows us to compare the findings of each specific haplogroup cluster to a mixed population of haplogroup clusters. This helps with interpreting the results especially since there is no strict biological evidence that defines one particular haplogroup cluster as a control or reference group. After determining clusters with statistically significant modifying effects on the PM2.5-DNAm-age relationship, we re-ran the models testing the modifying role of the individual haplogroups within those particular clusters and with individual PM2.5 components (sulfate and ammonium) as the predictors. Again, all of these models were fully-adjusted.

Mitochondrial DNA Copy Number as an Effect Modifier and/or Mediator—Unlike haplogroups, which are determined at birth and remain the same throughout life,

copy number can change throughout life. In fact, empirical evidence exploring the relationships between short-term versus long-term PM2.5 exposure and mitochondrial genome abundance suggest that copy number is subject to much change over time[45]. Moreover, there is experimental evidence demonstrating that depletion of the mitochondrial genome results in aberrant methylation of nuclear DNA at promoter CpG islands[46]. Given this evidence, we hypothesized that the association of PM2.5 with DNAm-age could be mediated through and/or modified by copy number. To test this hypothesis, we employed a 4-way decomposition mediation method. Standard methods of testing for effect modification operate under the assumption that the modifier is not on the casual pathway between the exposure and outcome. Thus, these results may be misleading if mediation is truly present and the candidate modifier is indeed on the causal pathway[47]. The 4-way decomposition method circumvents the risk of these potentially misleading results by allowing one to simultaneously parse out: 1) the controlled direct effect [the effect of the exposure on the outcome due neither to mediation nor interaction]; 2) the reference interaction [the effect of the exposure on the outcome due to interaction alone]; 3) the mediated interaction [the effect of the exposure on the outcome due to mediation and interaction]; and 4) the pure indirect effect [the effect of the exposure on the outcome due to mediation alone][48].

As mentioned earlier, there is some risk of simple interaction (effect modification) models resulting in misleading results if the candidate modifier is indeed on the causal pathway between the exposure and the outcome. This is not the case for simple mediation analyses because the goal of mediation is to provide evidence that a variable of interest is or is not on the causal pathway[47]. Due to the newness of the 4-way decomposition method, we performed a sensitivity analysis using a standard, simple mediation approach where we used fully-adjusted linear mixed-effects models and modeled[49–50]: Step 1) PM2.5 as a predictor of DNAm-age; Step 2) PM2.5 as a predictor of mitochondrial DNA copy number; and Step 3) PM2.5 as a predictor of DNAm-age controlling for mitochondrial DNA copy number. The proportion of the effect mediated by mitochondrial DNA copy number was calculated as the percentage of natural indirect effect over the sum of natural direct and natural indirect effect. In other words, [(Step 2 βPM2.5 * Step 3 βmtDNA CN)]/[(Step 2 βPM2.5 * Step 3 βmtDNA CN) + (Step 3 βPM2.5)]. The statistical significance of the mediation effect was assessed via the Sobel Z test.

Additional Sensitivity Analyses—Although all the covariates for diabetes, CHD, hypertension, and BMI were categorized using well-known and biologically relevant definitions, we performed sensitivity analyses examining if any resolution on potential confounding was lost by using these discrete categories. Specifically, we re-ran our direct association models and the simple mediation analysis (which specifically lists out mediation steps) using fully adjusted models where the aforementioned categorical variables were replaced with continuous measures of fasting blood glucose, total cholesterol, HDL cholesterol, systolic blood pressure, diastolic blood pressure, and BMI.

Our mediation analyses used fully-adjusted models; thus, we assumed limited or no exposure-outcome, exposure-mediator, and mediator-outcome confounding. However, due to the nature of this prospective repeated measures study, changes in DNAm-age at one visit could potentially affect copy number at a subsequent visit[51]. Hence, we performed an

analysis testing the aforementioned association to check the assumption of time-varying confounding.

Analysis Software—The 4-way decomposition mediation analysis was performed with a published SAS macro in SAS, version 9.3 (SAS Institute, Inc., Cary, North Carolina)[48]. All other 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 Statistics

Table 1 describes the demographic and clinical data for all participants. Participants had a mean (SD) DNAm-age of 74.1 (7.89) years and mean (SD) age of 74.8 (6.97) years. A majority of the men had completed at least 12 years of formal education (74%), consumed less than 2 drinks a day (81%), were former smokers (65%), and did not have coronary heart disease (65%) or diabetes (82%). The mean (SD) exposure levels for PM2.5, sulfate, and ammonium were 10.3 (2.13) $\mu g/m^3$, 3.39 (0.80) $\mu g/m^3$, and 1.04 (0.28) $\mu g/m^3$ respectively. Most participants were genotyped as having mitochondrial haplogroup cluster 2 (51%). 17% of the participants were cluster 1, 23% were cluster 3, and 9% were cluster 4. In regards to individual haplogroup frequencies, a majority of the participants were haplogroup V carriers (42%). Additional individual haplogroup frequencies are reported in Table S1.

3.2 One-Year Particle Levels and Haplogroup Clusters as Predictors of DNAm-age

Table 2 summarizes the results from fully-adjusted linear mixed-effects models examining the independent relationships of PM2.5, sulfate, ammonium, and individual haplogroup clusters with DNAm-age. One-year IQR increases in PM2.5 (p=0.007), sulfate (p<0.0001), and ammonium (p=0.0005) were all significantly associated with increases in DNAm-age of at least 0.58 years (approximately 7 months). None of the haplogroup clusters were significantly associated with DNAm-age.

3.3 Effect Modification by Haplogroup Clusters and Individual Haplogroups

Figure 1 depicts the modifying role of the mitochondrial haplogroup clusters on the association of one-year PM2.5 levels with DNAm-age. Only the cluster 2 mitochondrial DNA genotype significantly (p=0.007) modified the association of PM2.5 levels with DNAm-age. The effect of PM2.5 on DNAm-age was diminished by approximately 1 year when comparing individuals with the cluster 2 genotype to all individuals without the cluster 2 genotype. Figure 2 depicts a subsequent analysis examining the modifying role of the individual haplogroups in cluster 2 (haplogroups V and H) on the association of PM2.5 levels with DNAm-age. The effect of PM2.5 on DNAm-age was diminished by approximately 1 year when comparing individuals with the haplogroup V genotype to all individuals without the haplogroup V genotype (p=0.01). Figure 3 depicts the modifying role of haplogroup cluster V on the association of the PM2.5 components sulfate and ammonium with DNAm-age. Similar to total PM2.5, the association of ammonium with DNAm-age was diminished in individuals with a haplogroup V genotype when compared to individuals without a haplogroup V genotype (p=0.03). This relationship persisted even

when we included one-year nitrate levels as a covariate in the mixed-effects model (Figure S1).

3.4 Relationships of Mitochondrial DNA Copy Number with DNAm-age

In fully-adjusted linear mixed-effects models examining the relationship of copy number with DNAm-age, we found that copy number ($\beta = -3.31$, $p<0.0001$) was significantly, negatively associated with DNAm-age. However, copy number was not significantly associated with chronological age ($\beta=0.57$, $p=0.17$) (Table 3). These relationships persisted in sensitivity analysis adjusting for continuous variables instead of disease categories (Table S2).

3.5 Mediation Analyses

There was no evidence of time-varying confounding of DNAm-age on mitochondrial DNA copy number (Table S3). Table 4 presents the results of a 4-way decomposition meditation analysis examining the potential role of the mitochondrial DNA copy number as a mediator of the association of PM2.5 levels with DNAm-age. The controlled direct effect of one-year PM2.5 (due neither to mediation nor interaction) was positive and statistically significant ($\beta=0.81$, $p=0.02$). The pure indirect effect of one year PM2.5 (due to mediation alone) was also statistically significant ($\beta=0.22$, $p=0.02$). The percentage of the effect mediated by the copy number was estimated to be 12.2%. There was no evidence of any significant effect modification by copy number. These mediation relationships were consistent with results from the sensitivity analysis using the simple mediation approach (Table S4) and adjusting for continuous variables instead of disease categories (Table S5).

3.6 Relationships of Haplogroup Clusters with Mitochondrial DNA Copy Number

Table S6 presents the results of fully-adjusted linear mixed-effects models examining the association of each mitochondrial haplogroup cluster with mitochondrial DNA copy number. Only the association with cluster 3 was statistically significant ($\beta=0.06$, $p=0.02$). We also found that the association of PM2.5 with mitochondrial DNA copy number was greater in individuals with the haplogroup V genotype when compared to individuals without the haplogroup V genotype ($p=0.001$) (Figure S2).

4 Discussion

In the present study, we used fully-adjusted linear mixed-effects models to investigate the role of the mitochondrial genome in the relationship of long-term PM2.5 exposure with DNAm-age in a large longitudinal aging cohort. To our knowledge, this is the first study to demonstrate: 1) that mitochondrial DNA haplogroup V significantly reduces the association of one-year PM2.5 and ammonium exposure levels with DNAm-age and 2) that decreases in mitochondrial DNA copy number partially mediate the association of one-year PM2.5 exposure levels with DNAm-age. Additionally, we observed novel associations of mitochondrial DNA copy number with DNAm-age and the mitochondrial haplogroup cluster 3 genotype.

The number of studies examining relationships of ambient PM2.5 with DNAm-age are limited, but our results are consistent with what they report[8, 10, 52]. Here, we observed comparable, significant positive associations of PM2.5, sulfate, and ammonium with DNAm-age. No existing studies have examined the relationships of the mitochondrial genome with DNAm-age; however, we believed that such relationships would exist because both DNAm-age – as previously mentioned – and mitochondrial genome integrity are robustly associated with PM2.5 levels[31–32, 53].

In regards to mitochondrial haplogroups specifically, only two studies have examined relationships of mitochondrial haplogroups with air pollution and both studies examined the modifying role that mitochondrial haplogroups may have on the health effects of air pollution. The first study used a panel of 38 subjects with 417 total observations to test if air pollutant exposure-associated inflammation was stronger in carriers of mitochondrial haplogroup H versus U[54]. The authors justified limiting their study design to haplogroups H and U based on evidence suggesting that genetic variation due to mitochondrial haplogroups impacts the coupling of respiratory chain and the subsequent development of endogenous reactive oxygen species (ROS) by the mitochondria[55]. Haplogroup H has a relatively tightly coupled respiratory chain and has been associated with increased oxidative damage and risk of age-related diseases like Parkinson's[56–57]. On the other hand, haplogroup U has a less tightly coupled respiratory chain and has been shown to be protective against Parkinson's disease[56]. In the end, this study found that air pollutant (black carbon, carbon monoxide, nitric oxides and polycyclic aromatic hydrocarbons) associations with inflammatory markers (IL-6 and TNF-α) were stronger for individuals with the haplogroup H genotype when compared to haplogroup U individuals. Although this study's results were consistent with the aforementioned literature, the study was only based on 38 subjects and only relationships between two haplogroups were explored. In a larger study of 582 subjects with multiple visits, the researchers investigated if 9 different haplogroups (phylogenetically grouped into 4 clusters) resulted in differential susceptibility to cognitive effects of long-term black carbon exposure[37]. These researchers observed impaired cognition in carriers of cluster 1 (J and T) and even worse cognition of carriers of cluster 4 (I, W, and X). No effects were observed in cluster 2 (H and V) or 3 (K and U) carriers. Unlike the first study, these authors did not observe any effect modification in the clusters that contained haplogroups H and V. Another major difference between the two studies is that former reported significant findings with short-term air pollution exposures (≤ 5 days) and the second used a one-year exposure window. In all, the findings of these two studies suggest that the impact of haplogroups on air pollution relationships may be health outcome specific and may vary depending on the duration of air pollution exposure.

Given that DNAm-age has been associated with numerous age-related diseases, we believed that it would be associated with haplogroups that were also associated with age-related diseases, like haplogroup H. Nevertheless, we found no direct associations of haplogroups with DNAm-age in our study sample. However, we did find that haplogroup cluster 2 (V and H) significantly lessened the positive association of PM2.5 levels with DNAm-age. Further analyses suggested that this protective effect was predominately due to haplogroup V and it persisted even when examining the effects of the PM2.5 component ammonium. Although cluster 2 haplogroups, like H, have been traditionally thought to be health-adverse, there is

also existing evidence that these haplogroups may also offer some health benefits. For instance, haplogroup H carriers were found to have a 2.12 fold increased chance of survival at 180 days following a septic episode compared to non-carriers of haplogroup H[30]. In the sepsis study the researchers did not measure other haplogroups, but it is possible that related haplogroup V could also be protective if explored. Furthermore, in a study that compared the frequency distributions of haplogroups in athletes versus non-athlete controls, researchers found that the V haplogroup was overrepresented in endurance athletes (15.7%) compared with controls (7.5%)[58]. A major issue in existing haplogroup research is that groups being compared are not always the same and often relative findings are being interpreted. Thus, findings of an adverse effect of haplogroup H when it is compared to haplogroup U may not exist when haplogroup H is compared to haplogroup V. In an attempt to remedy future issues with such comparisons, our study always compares carriers of a specific haplogroup or cluster to all other individuals who were not carriers of the haplogroup or cluster. Thus, we are effectively comparing carriers of each haplogroup to a mixed population of haplogroups. Still, future studies using this comparison paradigm will be necessary to confirm our findings of a protective effect of cluster 2 and haplogroup V.

In respect to mitochondrial DNA copy number, our results agree with existing evidence that long-term PM2.5 exposure is associated with decreases in mitochondrial DNA copy number[6]. We also report novel evidence that mitochondrial DNA copy number is negatively associated with DNAm-age. Since mitochondrial copy number is viewed as a measure of the mitochondria's ability to respond to and buffer biological stressors, and a reduced copy number can be due to an exhausted mitochondrial buffering capacity (often observed with long-term environmental stresses)[31, 51], it is biologically conceivable that increases in buffering capacity would be associated with less of an "adverse" outcome like aging. Moreover, due to the strong associations of mitochondrial DNA copy number with DNAm-age and the known cross-talk between the nuclear and mitochondrial genomes[59], we believed that one measure may mediate the other's relationship with PM2.5. DNAm-age and mitochondrial DNA copy number were measured from blood taken at the same study visit so we took into account a number of considerations in assessing which would be the most biologically plausible outcome and the most biologically plausible mediator. First, although methylation of some nuclear genes like mitochondrial DNA polymerase γ catalytic subunit (PolgA) have been shown to regulate mitochondrial DNA copy number[60], PolgA methylation does not contribute to the DNAm-age metric[9]. Further, evidence has shown that DNAm-age is not simply the sum of its component CpG DNA methylation levels[8]. Rather, DNAm-age is a biomarker of aging with a unique balance of stability and responsiveness that allows it to simultaneously reflect past exposures[61] and predict future disease risk[62]. This unique balance of stability and responsiveness is best explained by the fact that, aside from the context of induced pluripotent stem cells, DNAm-age appears to only increase with time[63]. In the seminal DNAm-age paper by Horvath (2013), it is hypothesized that "DNAm age measures the cumulative work done by a particular kind of epigenetic maintenance system (EMS), which helps maintain epigenetic stability … This model would explain the high tick rate during organismal development since a high power is required to maintain epigenetic stability during this stressful time. At the end of development, a constant amount of power is sufficient to maintain stability leading to a constant tick rate … DNAm age

should be accelerated by many perturbations that affect epigenetic stability[9].” In line with this current understanding of DNAm-age, it is feasible that different environmental exposures or biological microenvironments that affect epigenetic stability could exacerbate the otherwise constant rate of DNAm-age increase[61, 64].

In contrast to DNAm-age, evidence shows that mitochondrial DNA copy number is a more variable metric able to rapidly change (increase or decrease) in response to short-term and long-term exposures but unable to intrinsically record long-term trends because it must be kept within a relatively stable range to maintain optimal physiological function[45]. Moreover, mitochondrial effectors like apoptosis-inducing factor, which are normally localized in the mitochondria, have been shown to translocate to the nucleus where they trigger DNA fragmentation, chromatin condensation, and other DNA changes[59]. Flavin adenine dinucleotide (FAD) and α ketoglutarate (α-KG) are two additional co-factors that are synthesized within the mitochondria, but are actively involved in the processes of nuclear methylation[65]. Thus, for our mediation analysis, we ultimately found that most evidence supported DNAm-age as an outcome with long-term reporting ability and a mitochondrial copy number as a mediator. Given this analytical framework, we found that mitochondrial DNA copy number significantly mediated about 12% of the observed effect of PM2.5 on DNAm-age. Mitochondrial DNA copy number has been shown to decrease with age and copy number has been associated with other age-related outcomes like frailty and mortality[66–67]. Hence, our findings are in agreement with existing aging research but controlled experiments must be performed to confirm if copy number is indeed a mediator of the relationship between PM2.5 and DNAm-age.

Finally, we explored the relationships of mitochondrial haplogroups and copy number in our study sample. The weaker association between copy number and DNAm-age in individuals with haplogroup V, may suggest that copy number is not the ultimate source of their protection against the effects of PM2.5. This theory is also supported by the findings that 1) copy number is not directly associated with haplogroup cluster 2 (V and H) and 2) copy number only mediates 12% of the association between PM2.5 and DNAm-age.

The current study possesses a number of strengths including the use of novel biomarker and a large longitudinal cohort with repeated measures of ambient pollutant exposures, DNA methylation, mitochondrial genome measures, and potential confounders. In fact, this is the first study to use mitochondrial genetic variants and genome abundance to study the relationship of ambient particles with DNAm-age. Still, our study has a few notable limitations. First, we utilized address-specific $PM_{2.5}$ and $PM_{2.5}$ component exposure estimates which could potentially misclassify personal exposure levels. However, the majority of NAS participants are retired and very likely spend most of their time at home. Moreover, any resulting non-differential misclassification is likely to attenuate statistical associations rather than bias them away from the null[68–69]. Secondly, the mitochondrial genotyping technique resulted in haplogroup designations that may not encompass more recent mutations that could potentially impact the relationship of PM2.5 with DNAm-age. Nonetheless, the haplogroups that were used have been utilized in many studies and our main objective was to identify common, normal forms of mitochondrial variation that may impact the PM2.5-DNAm-age relationship. Larger studies are warranted to evaluate the

impact of rarer forms of mitochondrial genome variation on the PM2.5-DNAm-age relationship. Finally, our findings are based on a cohort of elderly Caucasian males that reside in a lightly-polluted environment. Additional studies involving other demographic groups and in different environments will be needed to confirm our findings more broadly.

Overall, our study supports the premise that mitochondrial physiology is important for DNAm-age relationships, particularly in the context of ambient fine particle air pollution. Our data specifically suggests that mitochondrial haplogroups and copy number appear to be two different – but not necessarily mutually exclusive – ways that the relationship of PM2.5 with DNAm-age is impacted by mitochondrial physiology. Future research aimed at further understanding the relationships of mitochondrial physiology with shared PM2.5 and aging-related health outcomes will be critical for addressing this important public and environmental health topic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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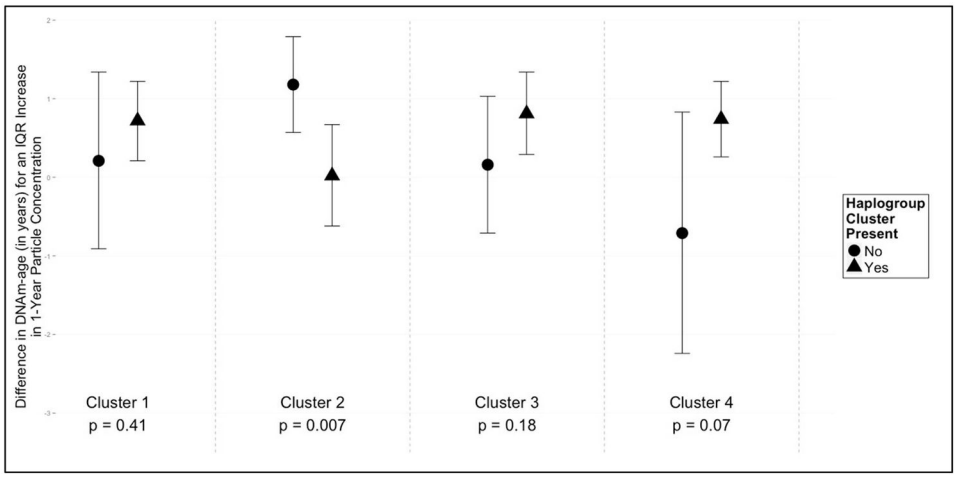

Figure 1.

Difference in DNAm-age for one interquartile range increase in one-year PM2.5 exposure comparing participants with and without the respective mitochondrial haplogroup clusters in fully-adjusted mixed-effects models. Cluster 1 (JT); Cluster 2 (VH); Cluster 3 (UK) and Cluster 4 (IWX).

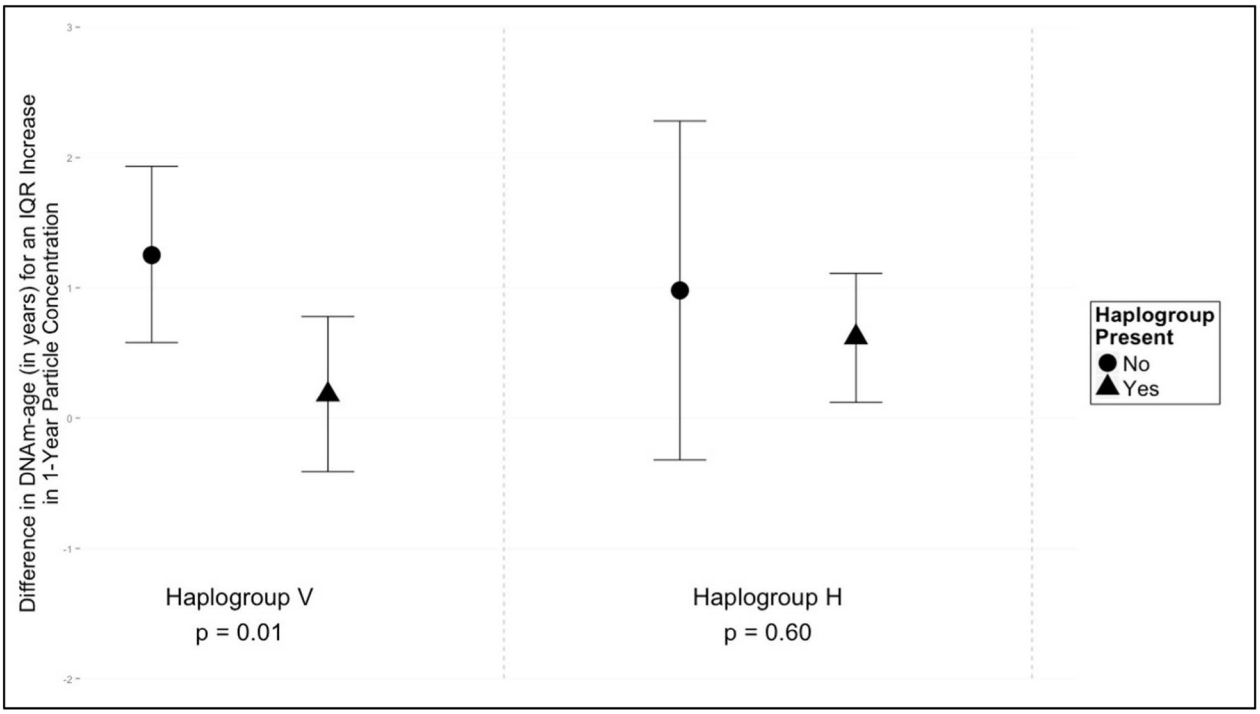

Figure 2.

Difference in DNAm-age for one interquartile range increase in one-year PM2.5 exposure comparing participants with and without the respective mitochondrial haplogroups from cluster 2 in fully-adjusted mixed-effects models.

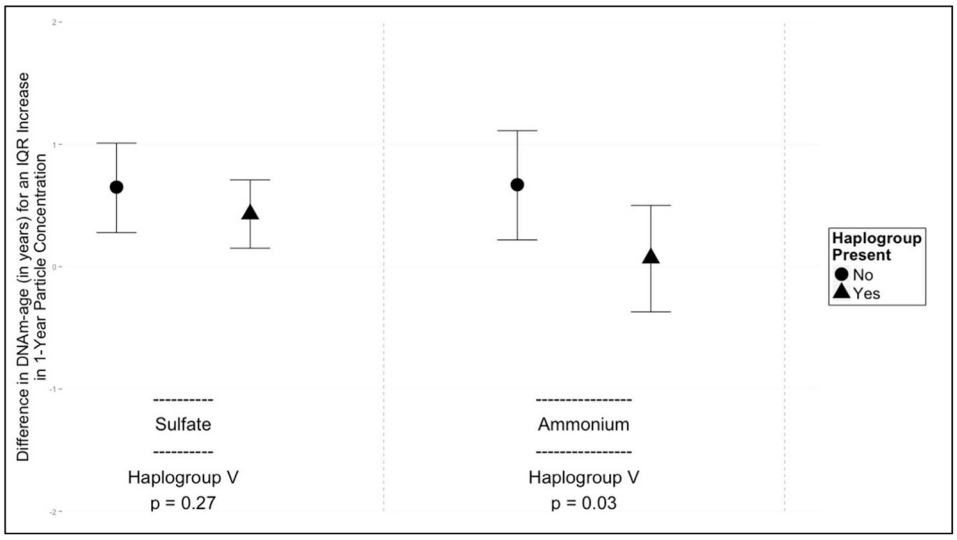

Figure 3.

Difference in DNAm-age for one interquartile range increase in one-year sulfate and ammonium exposure comparing participants with and without the V mitochondrial haplogroup in fully-adjusted mixed-effects models.

Table 1

Characteristics of Study Subjects (2000 – 2011)

Main Variables	All Visits, N = 870
Age (years), mean (SD)	74.8 (6.97)
DNAm-age (years), mean (SD)	74.1 (7.89)
One- Year Fine Particle Level (μg/m^3), mean (IQR)	
PM2.5	10.3 (2.13)
Sulfate	3.39 (0.80)
Ammonium	1.04 (0.28)
Mitochondrial Haplogroup Cluster, N (%)	
1 (JT)	146 (17)
2(VH)	447 (51)
3 (UK)	200 (23)
4 (IWX)	87 (9)
Lifestyle and Environmental Variables	
Alcohol Consumption, N (%)	
< 2 drinks/day	705 (81)
≥ 2 drinks/day	165 (19)
BMI, N (%)	
Healthy/Lean	206 (24)
Overweight	457 (52)
Obese	207 (24)
Education, N (%)	
≤ 12 years	224 (26)
12 – 16 years	402 (46)
> 16 years	244 (28)
Pack years, mean (SD)	20.9 (24.8)
Smoking Status, N (%)	
Current	40 (5)
Former	566 (65)
Never	264 (30)
Season, N (%)	
Spring	219 (25)
Summer	182 (21)
Fall	298 (34)
Winter	171 (20)
Temperature (°C), mean (SD)	11.3 (0.98)
Age-Related Diseases	
Coronary Heart Disease, N (%)	
Yes	308 (35)

Main Variables	All Visits, N = 870
No	562 (65)
Diabetes, N (%)	
Yes	159 (18)
No	711 (82)
Hypertension, N (%)	
Yes	639 (73)
No	231 (27)
Lifetime Cancer Diagnosis, N (%)	
Yes	486 (56)
No	384 (44)

Table 2

Mean One-Year Particulate Concentrations and Mitochondrial Haplogroup Cluster as Independent Predictors of DNAm-age (N = 870)

Predictor	Difference in DNAm-age for IQR (95% CI)	*P*
PM2.5	0.64 (0.18, 1.11)	0.007
Sulfate	0.58 (0.35, 0.82)	<0.0001
Ammonium	0.58 (0.26, 0.91)	0.0005
Haplogroup Cluster		
1 (JT)	−0.27 (−1.59, 1.05)	0.69
2 (VH)	−0.42 (−1.43, 0.60)	0.42
3 (UK)	0.93 (−0.28, 2.15)	0.13
4 (IWX)	−0.22 (−1.99, 1.55)	0.81

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 and ammonium models are additionally adjusted for total PM2.5 mass. Haplogroup models are adjusted for all three particles.

Table 3

Relationships of Mitochondrial Copy Number with Age and DNAm-age (N=797)

Outcome	Difference in Outcome for IQR (95% CI)	*P*
Age	0.57 (−0.25, 1.39)	0.17
DNAm-age	−3.31 (−4.62, −2.00)	<0.0001

All models adjusted for mitochondrial haplogroup, PM2.5, sulfate, ammonium, blood cell type, temperature, pack years, smoking status, season, BMI, alcohol consumption, education, lifetime cancer diagnosis, hypertension, diabetes, and coronary heart disease. DNAm-age model is also adjusted for chronological age.

Table 4

Results of 4-Way Decomposition Mediation Analysis of Mitochondrial DNA Copy Number as a Mediator of the Relationship of PM2.5 with DNAm-age (N=797)

Effect	Interpretation	β (95% CI)	SE	t	*P*	*P*mediation	% of Effect Mediated by Mediator
Controlled Direct Effect	Due neither to mediation nor interaction	0.81 (0.15, 1.48)	0.34	2.4	0.02	-	-
Reference Interaction	Due to interaction alone	0.12 (−0.04, 0.30)	0.08	1.53	0.13	-	-
Mediated Interaction	Due to mediation and interaction	−0.09 (−0.20, 0.02)	0.06	−1.63	0.10	-	-
Pure Indirect Effect	Due to mediation alone	0.22 (0.07, 0.38)	0.08	2.79	0.01	0.02	12.2

Results based on fully-adjusted 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. Effects were calculated using the published SAS macro where continuous covariates were set to their mean values and categorical variables were set to the category with the greatest proportion of study participants.