

# miRNA processing gene polymorphisms, blood DNA methylation age and long-term ambient PM<sub>2.5</sub> exposure in elderly men

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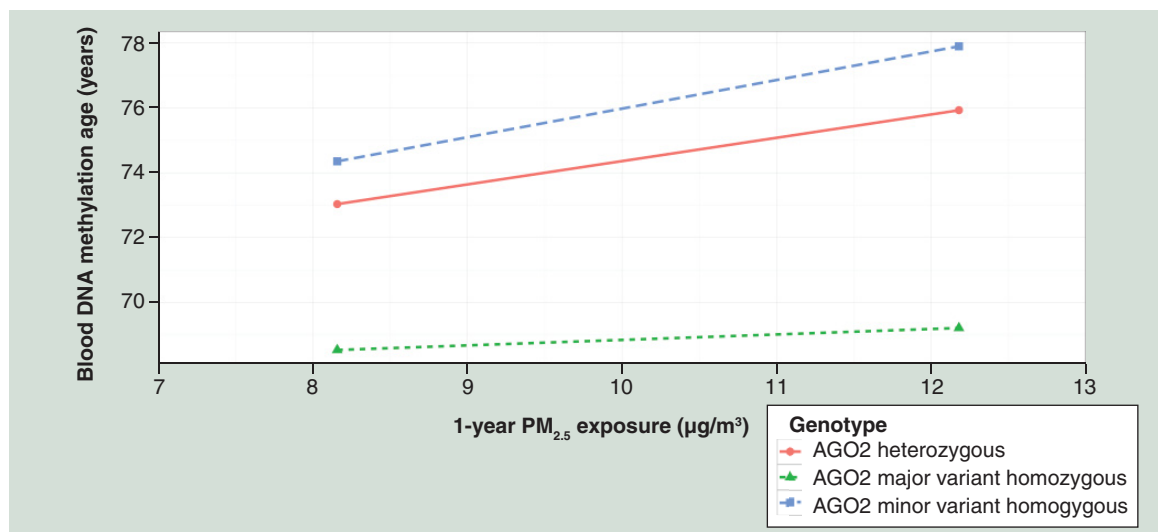
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**Aim:** We tested whether genetic variation in miRNA processing genes modified the association of PM<sub>2.5</sub> with DNA methylation (DNAm) age. **Patients & methods:** We conducted a repeated measures study based on 552 participants from the Normative Aging Study with multiple visits between 2000 and 2011 (n = 940 visits). Address-level 1-year PM<sub>2.5</sub> exposures were estimated using the GEOS-chem model. DNAm-age and a panel of 14 SNPs in miRNA processing genes were measured from participant blood samples. **Results & conclusion:** In fully adjusted linear mixed-effects models, having at least one copy of the minor rs4961280 [AGO2] allele was associated with a lower DNAm-age ( $\beta = -1.13$ ; 95% CI: -2.26 to -0.002). However, the association of PM<sub>2.5</sub> with DNAm-age was significantly ( $P_{interaction} = 0.01$ ) weaker in homozygous carriers of the major rs4961280 [AGO2] allele ( $\beta = 0.38$ ; 95% CI: -0.20 to 0.96) when compared with all other participants ( $\beta = 1.58$ ; 95% CI: 0.76 to 2.39). Our results suggest that miRNA processing impacts DNAm-age relationships.

Graphical abstract:

miRNA processing AGO2 polymorphism (rs4961280) modifies the association of long-term ambient fine particle exposure with blood DNA methylation age



The graph depicts lines from a fully adjusted linear regression model with fine particle exposure levels ranging from the tenth to the ninetieth percentile, all other continuous variables held constant at their means, and all other categorical variables held at their most frequent level.

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**Keywords:** AGO2 • air pollution • epigenetic aging • miRNA • PM<sub>2.5</sub> • SNPs

Between 2015 and 2050, the global percentage of individuals over the age of 60 is expected to almost double from 12 to 22% [1]. This shift in the age composition of the global population is particularly important because it will likely be accompanied by immense public health and economic burdens due to an unprecedented volume of aging-related diseases. For instance, in the USA alone, the 2017 disease prevalence and economic costs associated with Alzheimer's dementia were 5.5 million and \$259 billion. By 2050, those statistics are expected to be 16 million and \$1.1 trillion, respectively [2]. In an effort to curb these expanding disease and economic burdens, there has been an ever growing emphasis on research aimed at understanding biological aging and the factors that contribute to adverse aging-related health outcomes.

Ambient fine particle air pollution (PM<sub>2.5</sub>) – often considered the world's largest singular environmental health risk – is one potentially modifiable risk factor for aging-related diseases including cardiovascular disease, cognitive decline and cancer [3–6]. Of particular interest, is the association of long-term PM<sub>2.5</sub> exposure levels with DNA methylation (DNAm) age, a novel epigenome-wide DNA methylation-based measure of biological aging [7,8]. Like other biomarkers of biological aging, DNAm-age has been associated with all-cause mortality and aging-related diseases [9,10]. In contrast to other biomarkers of aging, researchers remain highly uncertain about what DNAm-age is capturing on a molecular physiological level [11]. By examining the relationship of DNAm-age with PM<sub>2.5</sub>, a widely studied exogenous exposure and aging risk factor, we can begin to understand more about DNAm-age physiology.

As previously mentioned, DNAm-age is derived from measurements of DNA methylation. DNA methylation is a biological process where methyl groups are added to DNA nucleotides and often result in changes in gene expression [12]. miRNAs are small noncoding RNA molecules that can also regulate gene expression and have been associated with PM<sub>2.5</sub>, aging/aging-related diseases and DNA methylation [13–15]. miRNAs are produced from nuclear transcripts that form hairpin structures. Following nuclear and cytoplasmic processing by a series of enzymes, miRNAs are incorporated into a structure called the RNA-induced silencing complex (RISC). RISC achieves post-transcriptional gene regulation by using one strand of the incorporated miRNA to target mRNAs via nucleotide complementary base pairing. Once the relevant mRNA is targeted, RISC inhibits subsequent protein production by inducing mRNA cleavage or by reducing translation of the mRNA molecule [16].

Despite existing knowledge of the role of miRNAs in PM<sub>2.5</sub> and aging biology, studies have not yet examined the role of miRNAs in the PM<sub>2.5</sub>–DNAm-age relationship. Given the shared role of miRNAs and DNA methylation in gene regulation [17], we hypothesized that miRNA physiology would be related to DNAm-age and may play a role in the relationship of PM<sub>2.5</sub> with DNAm-age. In the present study, we investigated if SNPs in miRNA processing genes modified the associations of long-term PM<sub>2.5</sub> and PM<sub>2.5</sub> component species (sulfate and ammonium) exposure with DNAm-age in participants of the elderly Normative Aging Study (NAS). Rather than simply testing all the SNPs in our panel, we utilized a methodical framework to identify and analyze significant SNPs and PM<sub>2.5</sub>–SNP interactions. We first employed an elastic net (penalized regression) selection model to identify SNPs in miRNA processing genes that were specifically important to DNAm-age. Subsequently, we used fully adjusted linear mixed-effects models to test for statistically significant direct associations of the elastic net selected SNPs and PM<sub>2.5</sub>–SNP interactions with DNAm-age. We also conducted a number of secondary analyses to better ascertain if particular PM<sub>2.5</sub> component species were responsible for the relationships we observed.

## Methods

### Study population

The US Department of Veterans Affairs (VA) NAS is a longitudinal study of aging that was established in 1963 and recruited male participants from the Greater Boston area that were free of any chronic disease [18]. The NAS is now a closed cohort, but every 3–5 years since recruitment, participants return for onsite, follow-up study

visits. During these recurring visits, participants undergo thorough physical examinations, report lifestyle practices via questionnaires, and provide biospecimens including blood. At recruitment, all participants provided written informed consent to the VA Institutional Review Board and were at least 18 years of age. The VA and Harvard T.H. Chan School of Public Health Institutional Review Boards granted human subjects approval (protocol 14027–102).

Our study sample is derived from all NAS men with continued study participation since the year 2000, when address-level PM<sub>2.5</sub> component species estimates became available. We started with a total of 552 participants with 940 study visits (observations) between the years 2000 and 2011 [8]. Of these 552 participants, 249 (45%) had one study visit, 218 (40%) had two study visits and 85 (15%) had three or more study visits. From this sample, we then excluded participants missing miRNA processing gene polymorphism data. This resulted in a final study sample of 471 participants with 808 total study visits. In the final study sample, 208 participants (44%) had one visit, 189 (40%) had two visits and 74 (16%) had three or more visits.

### Measuring DNA methylation & computing DNAm-age

We extracted DNA from whole blood provided by participants during NAS visits. After performing bisulfite conversion on the DNA (EZ-96 DNA Methylation Kit, Zymo Research, CA, USA), we performed methylation analysis using the Illumina HumanMethylation450 BeadChip platform (Infinium HD Methylation protocol, Illumina, CA, USA). To ensure a similar age distribution across chips/plates and minimize batch effects, we used a two-stage age-stratified algorithm to randomize samples and randomized chips across plates. For quality control purposes, we removed samples where more than 5% of probes had a beadcount less than 3 or more than 1% of probes had a detection p-value > 0.05. The remaining samples were preprocessed with Illumina-type background correction without normalization and normalized with dye-bias and BMIQ3 adjustments. Next, we generated methylation  $\beta$ -values, which represent the percentage of methylation for each of the approximately 480,000 CpG sites in the BeadChip array.  $\beta = \text{intensity of the methylated signal (M)} / [\text{intensity of the unmethylated signal (U)} + \text{intensity of the methylated signal (M)} + 100]$ .

DNAm-age was computed using the publicly available online calculator [19]. DNAm-age was derived from a penalized regression (an elastic net) run on numerous datasets of diverse cell and tissue types where CpG probes shared by both the Illumina HumanMethylation27 and HumanMethylation450 BeadChip platforms were regressed on a calibrated version of chronological age. Three hundred and fifty-three CpGs that correlated with age (193 positively and 160 negatively) were selected by the elastic net [11]. 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, bone, and brain [11].

### One-year address-level ambient fine particulate matter (PM<sub>2.5</sub>) exposure estimation

We focused on the 1-year PM<sub>2.5</sub> exposure window because existing literature demonstrates that it is robustly associated with DNA-age [7]. Furthermore, >90% of NAS participants are retired; thus, home address exposures are expected to be a good proxy for their individual ambient exposures. Using the GEOS-chem chemical transport model [20], we generated daily estimates at the 1 km × 1 km area resolution for total PM<sub>2.5</sub>. The GEOS-chem model is particularly useful because it allows us to predict PM<sub>2.5</sub> component species like ammonium and sulfate at the same 1 km × 1 km area resolution. Sulfate and ammonium are the major PM<sub>2.5</sub> component species that have been previously shown to be important in predicting DNAm-age [8]. After geocoding and linking participants' residencies to an area-level grid point, we assigned particle estimates to each participant's address. One-year total PM<sub>2.5</sub> and PM<sub>2.5</sub> component species exposure estimates were determined by averaging daily exposures for the 365 days prior to the day of each participants' NAS visit. Tenfold cross-validation demonstrated that the model performed well for PM<sub>2.5</sub> mass and its component species with R<sup>2</sup>s ranging from 0.70 to 0.88 [21].

### Genotyping miRNA processing gene polymorphisms

The panel of 24 miRNA processing gene SNPs examined in this study were selected from previous studies that investigated the association of miRNA processing gene SNPs with chronic aging-related diseases [22,23]. Some of these same SNPs have been shown to modify relationships of ambient air pollutants with aging-related disease [24,25]. We performed genotyping on DNA extracted from participants' blood. Multiplex PCR assays were designed with Sequenom SpectroDESIGNER software (Sequenom, Inc., CA, USA). The extension product was subsequently

spotted onto a 384-well spectroCHIP and analyzed in the MALDI-TOF mass spectrometer (Sequenom, Inc.). We duplicated the assay for 5% of the samples. All 24 SNPs analyzed for this study were successfully detected.

Following genotyping, we excluded ten SNPs for which the number of participants who were homozygous minor variant carriers was less than ten (rs595961 and rs636832 in *AGO1*; rs197388 and rs197414 in *DDX20*; rs417309 in *DGCR8*; rs3742330 in *DICER1*; rs2740348 and rs3744741 in *GEMIN4*; rs1106042 in *PIWIL1*) and one in which Hardy–Weinberg equilibrium was not met at the 0.05 level (rs10719 in *DROSHA*). This exclusion criterion has been utilized in already published studies that use this panel of SNPs [24,25]. The remaining 14 SNPs were used in the study analyses. Linkage disequilibrium of SNPs within the same gene was previously assessed using the LDPlotter tool [26] [25].

## Statistical analysis

### *Elastic net selection of miRNA processing gene SNPs*

The aim of the present study was to examine if SNPs in miRNA processing genes modified the association of long-term PM<sub>2.5</sub> and PM<sub>2.5</sub> component species levels with DNAm-age. In an effort to limit multiple comparisons; and identify specific miRNA processing gene SNPs that are important to DNAm-age, we first employed an elastic net (penalized regression) via the glmnet function in the R glmnet package. Our elastic net method was similar to that described by Lenters and colleagues [27] and the full documentation for running all aspects of the elastic net via glmnet is publicly available [28]. We have also used a comparable elastic net strategy in a previous publication [29]. In short, the elastic net regression linear models utilized a hybrid of ridge and LASSO penalty functions to determine which SNPs were important to DNAm-age. By combining both of these penalty functions, the elastic net is able to perform selection while allowing for the inclusion of highly related genetic variants [30,31]. In our case, the highly related variants were the panel of miRNA processing gene SNPs. Specifically, in our elastic net selection model, all 14 SNPs and their respective PM<sub>2.5</sub>–SNP interactions were regressed on DNAm-age. Chronological age (continuous), blood cell proportions (plasma cells, CD4<sup>+</sup> lymphocytes, CD8<sup>+</sup> lymphocytes, natural killer cells, monocytes and granulocytes) (continuous, determined via Houseman and Horvath methods [11,32]), average 1-year temperature (continuous address-specific satellite measurements [8]), cumulative cigarette pack years (continuous), smoking status (current, former or never), and season of visit (spring [March–May], Summer [June–August], Fall [September–November] and Winter [December–February]), were also included in the selection model as unpenalized variables. The existing air pollution, DNA methylation and DNAm-age literature have identified these variables as important potential confounders [7–8,33–34]. Cross-validation was performed to determine the optimal degree of penalization and the minimum mean-squared error of prediction from repeated tenfold cross-validation was used in the final elastic net selection model. miRNA processing gene SNPs with nonzero model coefficients were considered as ‘selected’ by the elastic net.

### *Covariates*

The direct relationships of the miRNA processing gene SNPs with DNAm-age and the role of these SNPs as modifiers of the association of PM<sub>2.5</sub> with DNAm-age were examined using fully adjusted linear mixed-effects models. These models included a random participant-specific intercept to account for correlation between repeated outcome measures resulting from having multiple study visits for participants. In the analyses using fully adjusted models, we controlled for all the variables used in the elastic net selection model as well as BMI (lean [ $<25$ ], overweight [25–30], obese [ $>30$ ]), alcohol intake (yes/no more than or equal to two drinks daily), maximum years of education (continuous), cancer (yes/no history of lifetime cancer diagnosis), ischemic heart disease (yes/no based on electrocardiogram, validated medical records or physical exam), diabetes (physician diagnosis or a fasting blood glucose  $>126$  mg/dl) and hypertension (yes/no antihypertensive medication use or systolic blood pressure  $\geq 140$  mmHg or diastolic blood pressure  $\geq 90$  mmHg).

### *Effect modification by & direct associations of miRNA processing gene SNPs*

Using the same covariates from the direct effect fully adjusted linear mixed-effects models, we evaluated if the miRNA processing gene SNPs selected by the elastic net modified the association of PM<sub>2.5</sub> with DNAm-age. Given a statistically significant modifying effect, we evaluated if the SNP of interest modified the associations of sulfate and ammonium with DNAm-age. In these analyses the reference group was participants who were homozygous for the major variant of the SNP. For instance, if we evaluated the modifying role of ‘SNP A’ on the association of PM<sub>2.5</sub> with DNAm-age, we compared participants genotyped as homozygous for the major variant of ‘SNP A’ against all

other participants (i.e., participants genotyped heterozygous or homozygous for the minor variant of ‘SNP A’). We conducted secondary analyses exploring trends in significant modifier effects across all three genotypes (homozygous major variant, heterozygous and homozygous minor variant). When we observed that a miRNA processing gene SNP significantly modified the relationship of a PM<sub>2.5</sub> component species with DNAm-age, we also conducted an additional sensitivity analysis. In this sensitivity analysis, we subtracted that component species from total PM<sub>2.5</sub> and re-evaluated effect modification by the SNP.

Also using fully adjusted linear mixed-effects models, we determined if elastic net-selected miRNA processing gene SNPs had direct associations with DNAm-age when modeled as joint predictors with PM<sub>2.5</sub> levels. We also performed a sensitivity analysis, examining these direct associations, where we subtracted component species from total PM<sub>2.5</sub> as previously described above.

### Network analysis

We used the publicly available GeneMANIA platform [35] to explore gene network relationships (co-expression, physical interactions and genetic interactions) between the genes encompassing the elastic net-selected SNPs and the 353 genes that contribute CpGs to the DNAm-age metric.

### Analysis software

The gene network analysis was performed using the Genemania plugin for Cytoscape. All remaining 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.

## Results

### Descriptive statistics

Table 1 summarizes the demographic and clinical characteristics of study participants across all study visits. All participants were Caucasian males with a mean (standard deviation [SD]) chronological age and DNAm-age of 75.0 (7.03) and 74.1 (8.02). A majority of the participants completed at least 12 years of formal education (74%), were former smokers (67%) and were overweight or obese (77%). In this study sample, the prevalences of ischemic heart disease, diabetes and hypertension were 35, 18 and 75%, respectively. The lifetime prevalence of a cancer diagnosis was 57%.

The mean (IQR) 1-year PM<sub>2.5</sub>, sulfate and ammonium levels were 10.3 (2.15) µg/m<sup>3</sup>, 3.39 (0.81) µg/m<sup>3</sup> and 1.05 (0.29) µg/m<sup>3</sup>. Supplementary Table 1 presents the Pearson correlation coefficients and the proportion of total PM<sub>2.5</sub> mass of GEOS-chem transport model-derived PM<sub>2.5</sub> component species across all study visits. Sulfate makes up the greatest proportion of PM<sub>2.5</sub> mass (33.2%), and ammonium makes up 10.2% of PM<sub>2.5</sub> mass. The correlation coefficients for sulfate and ammonium with total PM<sub>2.5</sub> mass are 0.30 (p < 0.0001) and 0.51 (p < 0.0001), respectively.

### Elastic net-selected miRNA processing gene SNPs

Supplementary Table 2 lists the 14 miRNA processing SNPs that were included in the elastic net selection model. Of these 14 SNPs, four (rs4961280 [AGO2], rs6877842 [DROSHA], rs910924 [GEMIN4] and rs784567 [TARBP2]) were selected by the elastic net with DNA methylation as the outcome.

Of the four elastic net-selected SNPs, only rs4961280 (AGO2) and rs784567 (TARBP2) were significantly associated with DNAm-age in fully adjusted linear mixed-effects models that included PM<sub>2.5</sub> levels as a covariate (Table 2). For rs4961280 (AGO2), individuals who had at least one copy of the minor SNP allele on average had a 1.13-year lower DNAm-age than individuals with the homozygous major variant (allele) genotype (p < 0.05). When we compared all three genotypes, on average, individuals who were homozygous for the minor rs4961280 (AGO2) variant (AA) had the lowest DNAm-age. Homozygous major carriers (CC) had the highest DNAm-age and heterozygous individuals (CA) had an intermediate DNAm-age (Supplementary Figure 1). The trend for this relationship was statistically significant (p = 0.04). For rs784567 (TARBP2), individuals who had at least one copy of the minor SNP allele on average had a 1.35-year lower DNAm-age than individuals with the homozygous major allele genotype (p = 0.04, Table 2). When we looked across all three rs784567 (TARBP2) genotypes, a trend similar to rs4961280 (AGO2) was observed, but the trend did not reach statistical significance (p = 0.08, Supplementary Figure 1). These relationships persisted in sensitivity analyses where the ammonium component was subtracted from total PM<sub>2.5</sub> mass (Supplementary Table 3).

Table 1. Characteristics of study participants (2000–2011).

Main variables	All visits, N = 808
Age (years), mean (SD)	75.0 (7.03)
DNAm-age (years), mean (SD)	74.1 (8.02)
1-year fine particle level ( $\mu\text{g}/\text{m}^3$ ), mean (IQR):	
– PM <sub>2.5</sub>	10.3 (2.15)
– Sulfate	3.39 (0.81)
– Ammonium	1.05 (0.29)
<b>Lifestyle and environmental variables</b>	
Alcohol consumption, N (%):	
– <2 drinks/day	647 (80)
– $\geq 2$ drinks/day	161 (20)
BMI, N (%):	
– Healthy/lean	189 (23)
– Overweight	427 (53)
– Obese	192 (24)
Education, N (%):	
– $\leq 12$ years	206 (26)
– 12–16 years	379 (47)
– >16 years	223 (27)
Pack years, mean (SD)	21.2 (24.7)
Smoking status, N (%):	
– Current	36 (4)
– Former	538 (67)
– Never	234 (29)
Season, N (%):	
– Spring	204 (25)
– Summer	175 (22)
– Fall	271 (33)
– Winter	158 (20)
Temperature ( $^{\circ}\text{C}$ ), mean (SD)	11.3 (0.99)
<b>Aging-related diseases</b>	
Ischemic heart disease, N (%):	
– Yes	287 (35)
– No	521 (65)
Diabetes, N (%):	
– Yes	148 (18)
– No	660 (82)
Hypertension, N (%):	
– Yes	604 (75)
– No	204 (25)
Lifetime cancer diagnosis, N (%):	
– Yes	458 (57)
– No	350 (43)

DNAm: DNA methylation; IQR: Interquartile range; SD: Standard deviation.

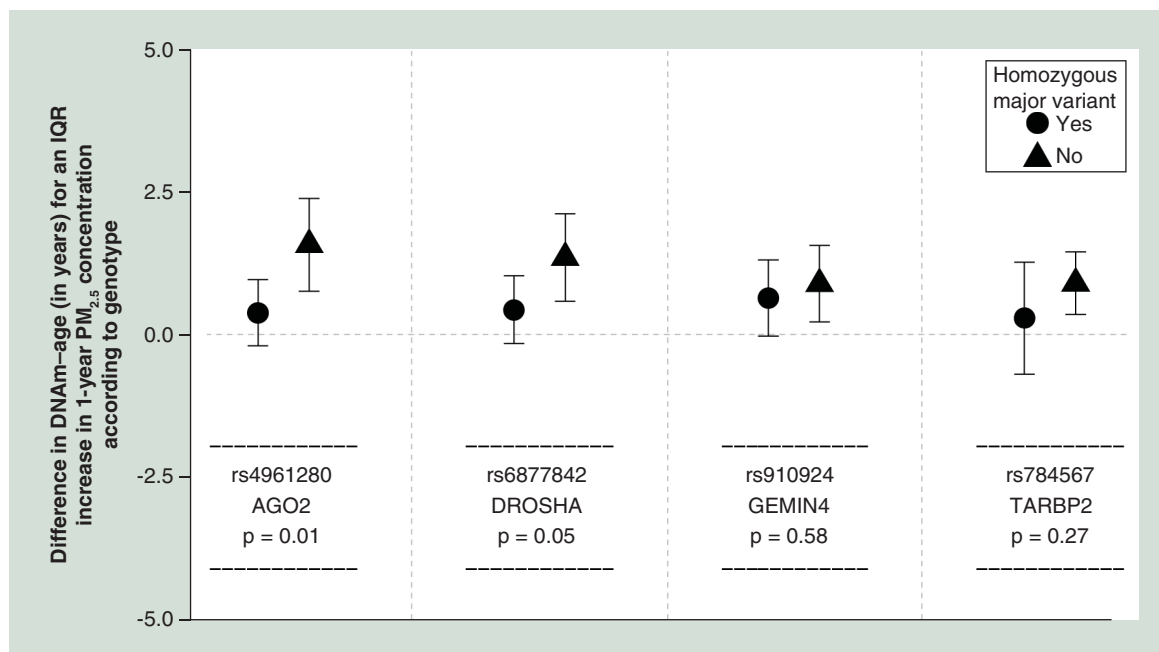
Out of all four SNPs, only the rs4961280 (*AGO2*) SNP significantly modified the association of PM<sub>2.5</sub> with DNAm-age ( $p = 0.01$ ) – although the rs6877842 (*DROSHA*) SNP neared statistical significance ( $p = 0.052$ , Figure 1). Specifically, the association of PM<sub>2.5</sub> with DNAm-age was greater in individuals who were not homozygous for the major *AGO2* variant (allele) when compared with individuals who were homozygous for the major allele. This trend was also observed for the ammonium PM<sub>2.5</sub> component species. No significant effect modification by the *AGO2* SNP was observed for sulfate levels and the rs6877842 (*DROSHA*) SNP did not significantly modify

**Table 2. Mean 1-year fine particle (PM<sub>2.5</sub>) concentrations and miRNA processing gene SNPs as joint predictors of DNA methylation age (n = 808).**

Predictor	Difference in DNAm-age for IQR (95% CI)	p-value
PM <sub>2.5</sub>	0.76 (0.24, 1.24)	<b>0.003</b>
Elastic net-selected miRNA SNPs <sup>†</sup>		
rs4961280 ( <i>AGO2</i> )	-1.13 (-2.26, -0.002)	<b>0.05</b>
rs6877842 ( <i>DROSHA</i> )	-0.78 (-1.92, 0.37)	0.18
rs910924 ( <i>GEMIN4</i> )	-0.41 (-1.47, 0.65)	0.45
rs784567 ( <i>TARBP2</i> )	-1.35 (-2.61, -0.09)	<b>0.04</b>

Note. Model adjusted for chronological age, blood cell type, temperature, pack years, smoking status, season, BMI, alcohol consumption, education, lifetime cancer diagnosis, hypertension, diabetes and ischemic heart disease.

<sup>†</sup> Values for the miRNA processing SNPs are in reference to participants whose genotypes are homozygous for the major variant. Bold text specifies statistically significant p-values (< 0.05). DNAm: DNA methylation; IQR: Interquartile range.



**Figure 1. Difference in DNA methylation age for one interquartile range increase in 1-year particle exposure levels comparing participants with and without a homozygous major variant genotype for *AGO2*, *DROSHA*, *GEMIN4* and *TARBP2* in fully adjusted linear mixed-effects models.**

the relationships of ammonium or sulfate with DNAm-age (Figure 2). We observed a significant ( $p = 0.01$ ) increasing trend for the association of PM<sub>2.5</sub> with DNAm-age when comparing the three rs4961280 (*AGO2*) SNP genotypes (Figure 3). The strongest (magnitude) association was observed in individuals who were homozygous for the minor allele and the smallest association was observed in individuals with the homozygous major allele genotype. An association of intermediate magnitude was observed in individuals who were heterozygous for the genotype. A similarly significant ( $p = 0.02$ ) trend across genotypes was observed for the relationship of ammonium with DNAm-age (Figure 3). In a sensitivity analysis where the ammonium component was subtracted from total PM<sub>2.5</sub> mass, we still observed a significant – although slightly attenuated – trend in the PM<sub>2.5</sub> (less ammonium) and DNAm-age relationship across *AGO2* genotypes (Supplementary Figure 2).

### Gene network analysis

Figure 4 depicts the results of a network analysis examining relationships of *AGO2*, *DROSHA*, *GEMIN4* and *TARBP2* with the 353 genes that contribute CpGs to the DNAm-age measure. *IPO8* was the sole DNAm-age CpG contributing gene that had a physical interaction with *AGO2*. Many genes were found to have genetic interactions

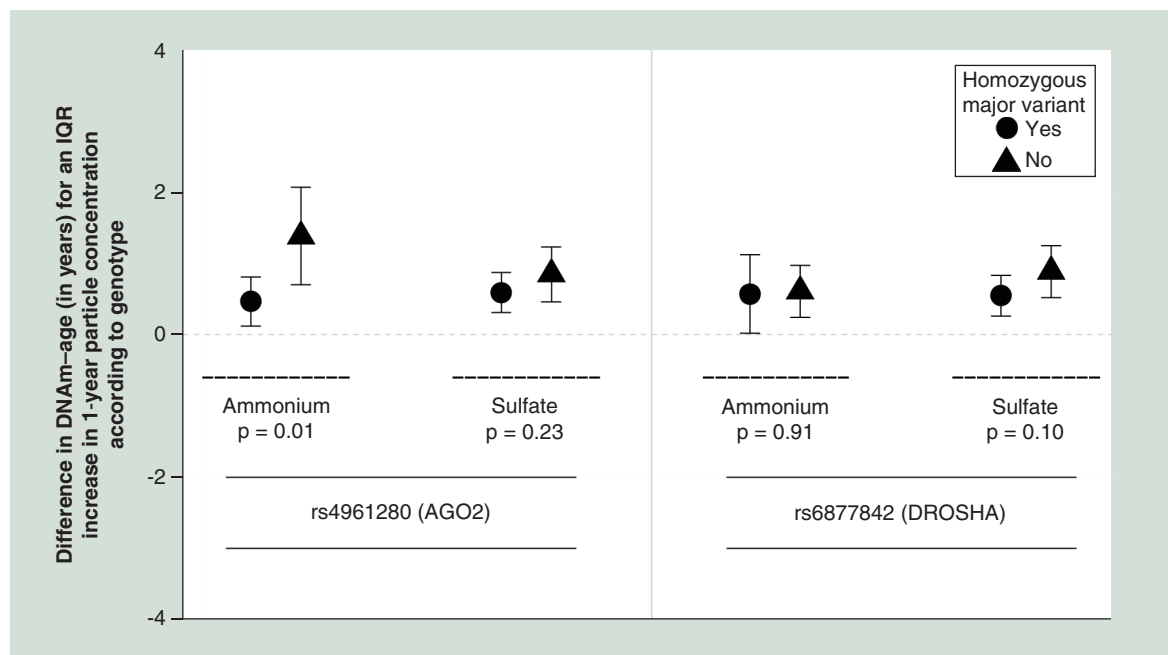


Figure 2. Difference in DNA methylation age for one interquartile range increase in 1-year particle exposure (ammonium and sulfate) levels comparing participants with and without a homozygous major variant genotype for *AGO2* and *DROSHA* in fully adjusted linear mixed-effects models.

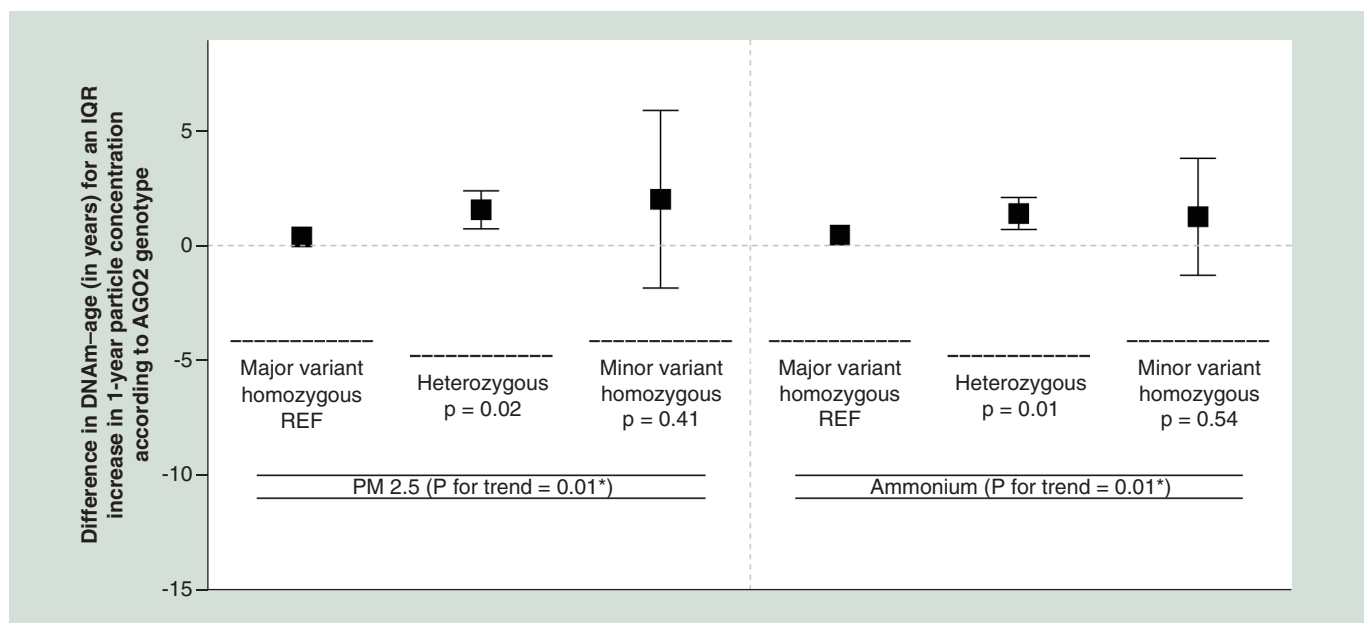
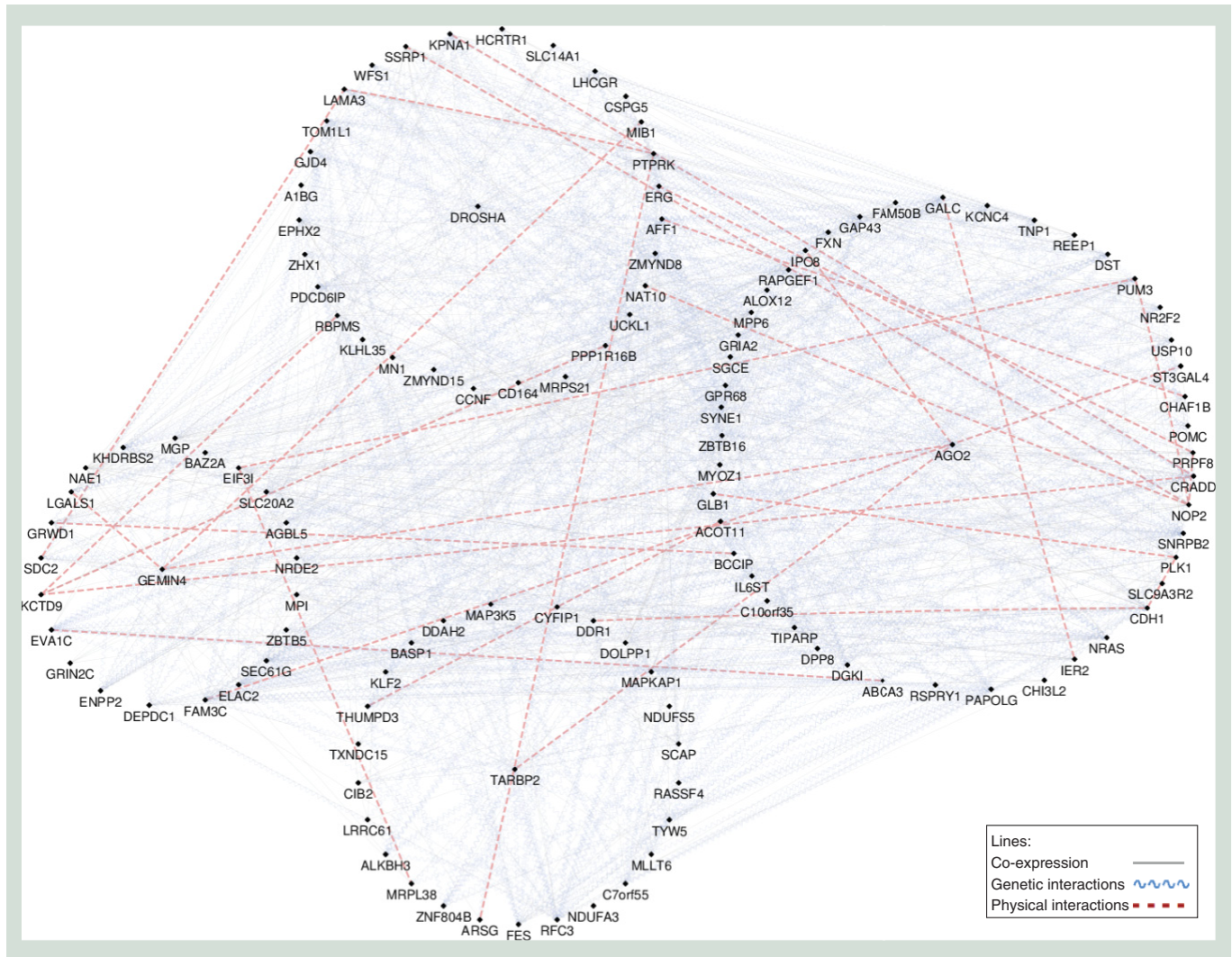


Figure 3. Difference in DNA methylation age for one interquartile range increase in 1-year particle exposure (PM<sub>2.5</sub> and ammonium) levels comparing participants of homozygous major variant (n = 526), heterozygous (n = 257) and homozygous minor variant genotypes (n = 25) for *AGO2* in fully adjusted linear mixed-effects models.

\*p-value for the test of linear trend across genotypes was based on a linear mixed-effects regression model where the three *AGO2* genotypes were fit as a continuous measure.

or be co-expressed with *AGO2*. *PAPOLG* and *TIPARP* were the only two genes that had both genetic interactions and were co-expressed with *AGO2*. *PAPOLG* and *TIPARP* were also co-expressed or had a genetic interaction with *DROSHA* (Supplementary Table 4).





**Figure 4.** Curated network map depicting relationships of *AGO2*, *DROSHA*, *GEMIN4* and *TARBP2* with genes that contribute component CpG methylation to DNA methylation age. Each of the elastic net-selected genes is surrounded by a circle of related genes that contribute CpG methylation to the DNAm-age metric. Solid lines that connect genes represent co-expression. Dashed lines that connect genes represent physical interactions. Squiggly lines that connect genes represent genetic interactions.

## Discussion

The present study utilized a DNAm-age elastic net selection model to identify four SNPs in miRNA processing genes (rs4961280 [*AGO2*], rs6877842 [*DROSHA*], rs910924 [*GEMIN4*] and rs784567 [*TARBP2*]) of which two (rs4961280 [*AGO2*] and rs784567 [*TARBP2*]) were directly associated with DNAm-age in a population of community-dwelling elderly men. Additionally, the study demonstrated a significant modifier effect of the rs4961280 (*AGO2*) SNP on the associations of 1-year PM<sub>2.5</sub> and ammonium (one PM<sub>2.5</sub> component species) levels with DNAm-age. More specifically, our data suggest that the association of PM<sub>2.5</sub> with DNAm-age is attenuated in individuals carrying at least one copy of the rs4961280 (*AGO2*) major variant allele. Our results were consistent (though slightly attenuated) in sensitivity analyses where we subtracted ammonium levels from total PM<sub>2.5</sub> mass. This suggests that the impact of the rs4961280 (*AGO2*) SNP on the relationship between PM<sub>2.5</sub> and DNAm-age is largely – but not exclusively – due to ammonium levels. Moreover, a gene network analysis revealed physical interactions, genetic interactions and co-expression relationships of *AGO2*, *DROSHA*, *GEMIN4* and *TARBP2* with genes that contribute CpGs to the DNAm-age metric. To our knowledge, this is the first study to examine relationships of miRNA processing physiology with epigenetic age both independently and in the context of long-term PM<sub>2.5</sub> exposure.

*DROSHA* is a gene located on human chromosome 5 and it encodes an RNA-specific endoribonuclease that is involved in the initial step of nuclear miRNA processing [36]. *GEMIN4* and *TARBP2* are located on chromosomes 17 and 12, respectively, and they both encode enzymes that are involved in the cytoplasmic processing of miRNAs. After a literature review examining the relationships of the SNPs in these three genes with air pollution, we found only one previous study – also in the Normative Aging Study cohort – demonstrating that in comparison to other participants, individuals heterozygous for the rs910924 (*GEMIN4*) SNP genotype were more likely to have lower global cognition measurements given the same level of black carbon exposure [24]. Nonetheless, we did find a number of studies implicating *DROSHA*, *GEMIN4* and *TARBP2* in numerous aging-related diseases including prostate cancer and colorectal cancer [37,38]. Since cancers are often related to changes in DNA methylation, the connections of these genes with cancer may be the reason why their SNPs were selected by the elastic net [39].

In contrast to the other three SNPs, we did observe significant effect modification of the PM<sub>2.5</sub>–DNAm-age relationship by the rs4961280 (*AGO2*) SNP. Furthermore, we also observed a significant direct relationship of the rs4961280 (*AGO2*) SNP with DNAm-age. *AGO2* (Argonaute Protein 2) is a gene located on human chromosome 2. Argonaute (AGO) proteins, including AGO2, form the core of the RNA-induced silencing complex (RISC) which is involved in gene silencing via RNAi. AGO proteins are well conserved across species and structurally include an amino-terminal, PAS, Piwi, and MID domains. Humans have eight AGO proteins; however, only AGO 1–4 are capable of loading miRNA in RISC. Moreover, only AGO2 appears to have the ability to cleave mRNA targets and achieve transcript instability/silencing [40]. In addition to their role in RISC, it has also been demonstrated that AGO proteins play a role in stabilizing and maintaining proper levels of mature miRNA strands [41].

Our data suggested that individuals with at least one copy of the rs4961280 (*AGO2*) major variant had an attenuated association of PM<sub>2.5</sub>/ammonium with DNAm-age when compared with individuals who were homozygous for the minor variant. However, individuals with at least one copy of the minor variant on average had lower DNAm-ages when compared with individuals that were homozygous for the major variant. Very few studies have explicitly examined relationships of the rs4961280 (*AGO2*) SNP and none of them were in the contexts of PM<sub>2.5</sub> or aging. However, we did find one study that demonstrated that the minor variant was associated with a reduced risk of benign prostatic hypertrophy (BPH) in a Serbian population [37]. Since BPH is most common in aging men, this study is in alignment with our finding that the minor allele is associated with qualities of being ‘younger’ (i.e., a lower DNAm-age or a lower risk of BPH) [42]. Further work will need to be done to understand why it is the major allele that attenuates the positive association of PM<sub>2.5</sub> with DNAm-age, but the minor allele that is directly associated with a lower biological age. Moreover, it will be helpful for the field to understand why ammonium relationships with DNAm-age were impacted by the rs4961280 (*AGO2*) SNP and sulfate relationships were not.

Of the existing studies that examine relationships of *AGO2* – not simply the SNP, but the gene – with aging in humans, the majority are cell culture based and describe *AGO2* as a factor involved in molecular processes related to biological aging such as cellular senescence, stem cell renewal and endothelial function [43–45]. We also found a few animal studies that showed relationships of *AGO2* with chronological aging. A study examining relationships of miRNAs with aging in *Drosophila* revealed that with age, there was a global increase in miRNAs loaded in *AGO2* but not *AGO1*. Furthermore, mutations in *AGO2* resulted in shorter life span and neurodegeneration. Together, these data suggest that *AGO2* impacts aging-associated processes [46]. Another study looking to elucidate how intermittent fasting increases longevity in *Caenorhabditis elegans* demonstrated that fasting upregulates the expression of miRNA-induced silencing complex (RISC) components including AGOs. In this study, fasting upregulated *AGO2* by twofold [47].

Our network analyses of *AGO2*, *DROSHA*, *GEMIN4*, *TARBP2* and genes that contribute CpGs to the DNAm-age metric demonstrated one physical interaction between *AGO2* and a gene called *IPO8*. *IPO8* (Importin 8) is a gene on chromosome 12 that encodes a protein involved in mediating the nuclear import of other proteins with nuclear localization signals. *IPO8* has also been shown to mediate the cytoplasm to nucleus transport of mature miRNAs. Moreover, this *IPO8*-mediated transport of miRNAs is dependent on the physical association of *IPO8* with the *AGO2* complex [48]. The literature primarily describes *IPO8* as an optimum reference gene for microarray and RT-PCR studies in multiple tissue types including the lung [49]. Two genes (*PAPOLG* and *TIPARP*) had both genetic interactions and co-expression relationships with *AGO2*. These two genes also had genetic interactions with *TARBP2* and *DROSHA*. *PAPOLG* (Poly [A] polymerase  $\gamma$ ) is a gene on chromosome 2 that encodes an exclusively nuclear-localized poly (A) polymerase responsible for catalyzing template-independent extension of the 3' end of a strand of DNA/RNA [50]. To our knowledge, no explicit studies have examined the relationships of *PAPOLG*

and biological aging but *PAPOLG* has been implicated in relationships involving aging-related health outcomes. For instance, a 12-week trial examined if a dietary intervention of 400 g/week of high-glucoraphanin broccoli altered plasma metabolites linked to cancer risk when compared with diets of 400 g/week of standard broccoli or 400 g/week of peas. No other modifications were made to the participants' diets. The study revealed that the levels of plasma metabolites (including flavin adenine dinucleotide [FAD]) of individuals receiving high-glucoraphanin broccoli were differentiated by *PAPOLG* genotypes. This suggests that *PAPOLG* may interact with diet to impact the levels of metabolites including those that have been implicated in cancer risk [51]. *TIPARP* (tetrachlorodibenzo-p-dioxin-inducible poly [ADP-ribose] polymerase) is a gene on chromosome 3 that encodes a member of the poly (ADP-ribose) polymerase super family [52]. In a study exposing human aortic endothelial cells to 10 µg/ml of fine and ultrafine ambient particulate matter from California, mRNA levels of enzymes including *TIPARP* increased [53]. Another study exposed human adenocarcinomic human alveolar basal epithelial (A549) cells to 10 µg/ml of winter and summer PM<sub>2.5</sub> from Milan and found that PM<sub>2.5</sub> from both seasons modulated *TIPARP* gene expression [54].

Strengths of our study include the combination of a novel biomarker, rigorous statistical methods and access to a large cohort with extensive and repeated information regarding PM<sub>2.5</sub> exposure levels, DNA methylation data and potential confounders from multiple study visits. This is the first study to use miRNA processing gene variants to study the relationship of ambient fine particles with DNAm-age. However, our study does have some notable limitations. First, we use a validated chemical transport model to generate address-level 1-year PM<sub>2.5</sub>, sulfate, and ammonium exposure estimates. Given that most NAS participants are retired, we believe that particle exposure levels at their homes approximately capture their personal exposures. Still, there is some risk of exposure misclassification. Nonetheless, such nondifferential misclassification is likely to underestimate any observed associations rather than bias them away from the null [55]. Second, we utilize a panel of a miRNA processing gene SNPs that is somewhat limited because it does not provide genome-wide resolution of all genes involved in miRNA processing. Nevertheless, this panel has been successfully utilized in other environmental health studies and we use a rigorous elastic net approach to identify our variants of interest [27]. Although we did not test for effect modification with all the SNPs in our panel, our targeted approach identified significant interactions that persisted even in sensitivity analyses. These findings will be informative to more comprehensive, future research. Last, our study examines the role of the miRNA processing pathway by using genetic variants of miRNA processing genes and our findings are based on a cohort of elderly Caucasian males who reside in a lightly polluted environment. Future studies involving other demographic groups, in different environments and using miRNA expression levels will be necessary to broadly confirm and add to these important but early findings.

## Conclusion

In conclusion, genotypes of the rs4961280 (*AGO2*) miRNA processing SNP were directly associated with DNAm-age and modified the associations of 1-year PM<sub>2.5</sub> and ammonium levels with DNAm-age in this population of community-dwelling Caucasian elderly men. Although our findings need to be confirmed in other individuals of this same demographic group and different populations, they begin to address the important research gap concerning the biological relevance of DNAm-age and the physiology of the PM<sub>2.5</sub>–DNAm-age relationship. Future studies will be necessary to elucidate more nuanced relationships of miRNA physiology with epigenetic aging.

## Data availability

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

## Financial & competing interests disclosure

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### Summary points

- Since miRNAs are heavily involved in gene regulation, we investigated the modifying role of genetic variation in miRNA processing genes on the PM<sub>2.5</sub>-DNA methylation (DNAm)-age relationship.
- After identifying SNPs important to DNAm-age with an elastic net, we tested for PM<sub>2.5</sub>-SNP interactions associated with DNAm-age.
- Four SNPs (rs4961280 [AGO2], rs6877842 [DROSHA], rs910924 [GEMIN4] and rs784567 [TARBP2]) were selected by the elastic net.
- Only the rs4961280 [AGO2] SNP modified the PM<sub>2.5</sub>-DNAm-age relationship.
- In fully adjusted linear mixed-effects models, the association of PM<sub>2.5</sub> with DNAm-age was significantly ( $P_{interaction} = 0.01$ ) weaker in homozygous carriers of the major AGO2 allele ( $\beta = 0.38$ ; 95% CI: -0.20, 0.96) when compared with all other participants ( $\beta = 1.58$ ; 95% CI: 0.76, 2.39).
- A gene network analysis revealed known physical, genetic and co-expression relationships of AGO2 with genes that contribute methylation values to the DNAm-age measure including *IPO8*, *PAPOLG* and *TIPARP*.
- We observed no effect modification by the other SNPs.
- Our results suggest that miRNA processing impacts DNAm-age relationships particularly in the context of long-term PM<sub>2.5</sub> exposure.

any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

### Ethics approval

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

### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: [www.futuremedicine.com/doi/full/10.2217/epi-2017-0094](http://www.futuremedicine.com/doi/full/10.2217/epi-2017-0094)

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