Health Effects of PM2.5 and Its Components on Mortality, Blood Pressure, and DNA Methylation

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Mortality, Blood Pressure, and DNA Methylation

Abstract

Epidemiological studies have examined the association between $PM_{2.5}$ mass and mortality, but there remains uncertainty about the relative importance of species. $PM_{2.5}$ contains various species, such as organic carbon, elemental carbon, and metals. Determining the differential toxicity of $PM_{2.5}$ species and identifying species with greatest toxicity is of great importance to emission-control strategies and regulations.

In the dissertation thesis, effects of $PM_{2.5}$ species on health outcomes on different levels were estimated. The first study examined the association between $PM_{2.5}$ species and mortality on approximately 4.5 million deaths for all causes, cardiovascular diseases, myocardial infarction, stroke, and respiratory diseases in 75 U.S. cities for 2000-2006, using city-season specific Poisson regression and multivariate meta-regression controlled for infiltration. Since cardiovascular diseases are leading causes of death within U.S. population, the second study aimed to determine which $PM_{2.5}$ species are associated with blood pressure, an indicator of cardiovascular health, in a longitudinal cohort. Linear mixed-effects models with the adaptive LASSO penalty were applied to longitudinal data from 718 elderly men in the Veterans Affairs Normative Aging Study (NAS),

1999-2010. Species considered included 8 metals (Fe, K, Al, Ni, V, Cu, Zn, and Na) and 3 non-metals (S, Si, and Se). At last, the relationship between long-term exposure to $PM_{2.5}$ species and epigenome-wide DNA methylation at 484 613 CpG probes in the longitudinal NAS cohort that included 646 subjects were investigated to explore the potential biological mechanisms on the epigenetic level in study 3.

The studies have showed an increased risk of mortality and blood pressure associated with $PM_{2.5}$, which varied with species, and differential DNA methylation linked to long-term exposure to particular components of $PM_{2.5}$. In conclusion, mass alone might not be sufficient to evaluate the health effects of particles. Understanding the toxicity of particle components is crucial to public health.

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Study 1. Associations of Fine Particulate Matter Species with Mortality in the United States: A Multicity Time-Series Analysis

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Abstract

Background: Epidemiological studies have examined the association between $PM_{2.5}$ and mortality, but there remains uncertainty about the seasonal variations in $PM_{2.5}$-related effects and the relative importance of species.

Objectives: to estimate the effects of $PM_{2.5}$ species on mortality and how infiltration rates may modify the association.

Methods: Using city-season specific Poisson regression, we estimated $PM_{2.5}$ effects on approximately 4.5 million deaths for all causes, CVD, MI, stroke, and respiratory diseases in 75 U.S. cities for 2000-2006. We added interaction terms between $PM_{2.5}$ and monthly average species-to-$PM_{2.5}$ proportions of individual species to determine the relative toxicity of each species. We combined results across cities using multivariate meta-regression, and controlled for infiltration.

Results: We estimated a 1.18% [95% confidence interval (CI): 0.93, 1.44%] increase in all-cause mortality, a 1.03% (95% CI: 0.65, 1.41%) increase in CVD, a 1.22% (95% CI: 0.62, 1.82%) increase in MI, a 1.76% (95% CI: 1.01, 2.52%) increase in stroke, and a 1.71% (95% CI: 1.06,

2.35%) increase in respiratory deaths in association with a 10-μg/m^3 increase in 2-day averaged $PM_{2.5}$ concentration. The associations were largest in the spring. Silicon, calcium, and sulfur were associated with more all-cause mortality, while sulfur was related to more respiratory deaths. County-level smoking and alcohol were associated with larger estimated $PM_{2.5}$ effects.

Conclusions: Our study showed an increased risk of mortality associated with $PM_{2.5}$, which varied with seasons and species. The results suggest that mass alone might not be sufficient to evaluate the health effects of particles.

Introduction

Over the past few decades, there has been much research on the adverse effects of ambient particulate matter (PM). A number of studies have used fine PM ($PM_{2.5}$, particles < 2.5 μm in aerodynamic diameter) as an exposure metric and estimated the effects of $PM_{2.5}$ on human health (Laden et al. 2006; Ostro et al. 2006; Pope and Dockery 2006; Zanobetti and Schwartz 2009). Meanwhile, researchers have found some $PM_{2.5}$ species significantly modify $PM_{2.5}$-related effects (Franklin et al. 2008; Lippmann et al. 2006; Zanobetti et al. 2009). $PM_{2.5}$ consists of many chemical components that originate from various sources, such as traffic, biomass burning and coal combustion. The U.S. National Research Council has emphasized the importance of examining the risk of PM species (NRC 2004). Determining the differential toxicity of $PM_{2.5}$ species and identifying species with greatest toxicity is of great importance to emission-control strategies and regulations.

The U.S. Environmental Protection Agency (EPA) established the $PM_{2.5}$ Speciation Trends Network (http://www.epa.gov/ttnamti1/speciepg.html) in 2000. Speciation sampling was

conducted every third or sixth day, which limits statistical power for analysis of responses to acute exposure and also prevent the examination of e.g. two-day moving averages of exposure which most studies find more strongly associated with mortality and hospital admissions than single day exposures. As a result, there are a limited number of studies investigating the toxicity of $PM_{2.5}$ components. These investigations have reported numerous components that may be responsible for particle toxicity such as elemental and organic carbon, sulfate, nitrate, and metals, including zinc, nickel, iron, potassium, and chromium (Atkinson et al. 2010; Bell et al. 2009; Franklin et al. 2008; Ostro et al. 2006; Valdes et al. 2012; Zhou et al. 2011).

Recently, Krall et al. reported on the association of 1-day average concentrations of species from the speciation network and mortality in 72 cities for the years 2000-2005 (Krall et al. 2013). This paper addresses a similar question, but with the following differences. First, Krall et al. analyzed PM components without controlling for PM mass risks. As pointed out by Mostofsky et al. (Mostofsky et al. 2012), it is possible to find associations for components because they are highly correlated with mass, and not because they are themselves particularly toxic. Second, it focuses on single day exposures. $PM_{2.5}$ mortality studies have consistently reported that the associations are spread over more than one day. Thus when one uses separate time series for components which are measured only 1 day in 6 or 1 day in 3, this will bias downward estimates, possibly more for some components than others. In addition, the loss of two thirds to five sixths of the data substantially reduces power.

US adults, particularly the elderly who dominate mortality statistics, spend approximately 90% of their time indoors (U.S.EPA 1989). While particles penetrate indoors, the infiltration rates vary with the extent to which windows and doors are open, which in turn can vary with local temperature and may therefore modify the association. Previous studies have reported such

modification (Franklin et al. 2008; Stafoggia et al. 2008; Zanobetti et al. 2009). In this paper we address these issues and in addition examine more species, add an additional year of observation, and look at specific causes of death.

Methods

Study sites

We included 75 U.S. cities in our study. Cities of interest were selected based on the availability of daily mortality, $PM_{2.5}$ mass, and speciation data for at least 400 days between 2000 and 2006.

Environmental data

We conducted county-level analysis for most cities as the city lies within a single county, and used multiple counties for a city whose population extends beyond the boundary of one county (Zanobetti and Schwartz 2009). We obtained $PM_{2.5}$ mass and species concentration data from the U.S. EPA Air Quality System Technology Transfer Network (http://www.epa.gov/ttn/airs/airsaqs/). $PM_{2.5}$ mass samples were collected daily in most of the cities, while the speciation monitoring sites were operated on a 1-in-3 or 1-in-6 day schedule. Most of the cities had a single monitor. For cities with more than one sampling site concentration data were averaged. Our analysis focused on organic carbon (OC), elemental carbon (EC), sodium (Na), aluminum (Al), silicon (Si), sulfur (S), potassium (K), calcium (Ca), vanadium (V), iron (Fe), nickel (Ni), copper (Cu), and zinc (Zn), because these species have been shown to be representative of several sources (e.g., motor vehicles, oil combustion, coal combustion, wood burning, sea salt,

and road dust) and their concentration levels are mostly above the method detection limits (Hopke et al. 2006). Furthermore, they have been studied by previous epidemiologic and toxicological studies (Bell et al. 2009; Franklin et al. 2008; Ostro et al. 2006; Zanobetti et al. 2009; Zhou et al. 2011). Monthly average proportions between each component and $PM_{2.5}$ mass were calculated for each city by dividing monthly concentrations of species by the respective mass mean.

Daily mean temperature in every city was obtained from the National Oceanic and Atmospheric Administration (http://www.noaa.gov/). We used 24-hour average temperature data from the closest weather station to the center of the city. Percent green space data were obtained from the National Land Cover Database, Multi-Resolution Land Characteristics Consortium (http://www.mrlc.gov/).

Health data

Daily mortality data were obtained from National Center for Health Statistics (http://www.cdc.gov/nchs/). We examined non-accidental deaths due to all causes and specific diseases, which were derived from the *International Statistical Classification of Disease*, 10th Revision (WHO 2007) codes as follows: all causes (ICD-10, A00-R99), cardiovascular diseases (ICD-10, I01–I59), respiratory diseases (ICD-10, J00–J99), myocardial infarction (ICD-10, I21-I22), and stroke (ICD-10, I60-I69).

We investigated several behavioral and other risk factors that have been reported to impact health (Baja et al. 2010; Dogra et al. 2007; Dwyer-Lindgren et al. 2013; Mora et al. 2007), including diabetes, being overweight or obese (i.e., BMI $\geq$ 25), smoking, quitting smoking, alcohol

consumption (having > two drinks per day), asthma, and leisure time physical activity, from the Behavioral Risk Factor Surveillance System (BRFSS) (CDC 2006). We applied county-level weighting methodology to obtain county-level percentages of these variables in 2006. For counties that were not available, we used data from the closest metropolitan or micropolitan statistical area (MMSA) and applied MMSA-level weighting methodology.

Statistical methods

We applied a two-stage analysis in our study. In the first stage, a city-specific season-stratified time-series analysis using Poisson regression in a generalized additive model (GAM) was used to estimate the association between daily mortality and the mean of $PM_{2.5}$ mass on the day of death and the day before death in each city and each season (defined as Spring: March – May; Summer: June – August; Fall: September – November; Winter: December – February). We controlled for time trend with a natural cubic regression spline with 1.5 degrees of freedom (d.f.) per season per year, for day of the week with indicator variables, and for daily temperature on the same day (lag 0) and on the previous day (lag 1) with a natural cubic spline with 3 d.f. for each. For every species, we calculated the monthly average species-to-$PM_{2.5}$ proportions for each month as a solution to the missing speciation data problem due to the 1-in-6 or 1-in-3 day sampling frequency. We then added, one at the time, the interaction terms between $PM_{2.5}$ and the monthly average species-to-$PM_{2.5}$ proportions of each individual species (Valdes et al. 2012). The model is as below:

$$\text{Log}E(Y_t) = Intercept + ns(time, df) + ns(temperature_t, df) + ns(temperature_{t-1}, df) + day\ of\ the\ week + \alpha Z_{t-1,t} + \beta p^i + \gamma Z_{t-1,t} p^i \quad [1.1]$$

where, $E(Y_t)$ is the expected death count at day t, ns is the natural cubic splines, $Z_{t-1,t}$ indicates 2-day averaged concentration of $PM_{2.5}$ at day $t-1$ and t, and p^i is the mean monthly proportion of species i to mass.

By using an interaction with the monthly mean ratio we avoid losing most of the daily observations, since we are able to use more than one day's exposure, and control for PM mass. While the use of the monthly ratio introduces some error in that variable, much of the variation in species mass is across cities, and between months within cities. For example, organic carbon, sulfate and nitrate are products of photochemical reactions whose rates are temperature-dependent, and this varies substantially across the U.S. and differently by month in different locations (Baker and Scheff 2007; de Gouw et al. 2005). It is important to note that if a species ratio is not significant in this analysis that does not mean that the species has no effect, it means its effect is not different than the average PM effect. A species with low or no toxicity would be expected to have a significant negative interaction term.

In the second stage of the analysis, we conducted a multivariate random effects meta-analysis and combined the 300 (i.e., 75 cities * 4 seasons) city-season specific effect estimates to obtain an overall association between $PM_{2.5}$ mass and its interaction with each species with mortality across all 75 cities:

$$\boldsymbol{Y}^i = \boldsymbol{X}\boldsymbol{B}^i \qquad [1.2]$$

where, $\boldsymbol{Y}^i$ is a (300 × 2) matrix, whose first column contains 300 city-season specific coefficients for $PM_{2.5}$ and the second column contains 300 city-season specific coefficients for interaction with species i, $\boldsymbol{X}$ is a (300 × 4) matrix for intercept, linear, quadratic and cubic temperature, and $\boldsymbol{B}^i$

indicates a (4 × 2) matrix of meta-regression coefficient for $PM_{2.5}$ and for interaction with species *i*.

It has been shown that high ventilation is seen at mild temperatures whereas low ventilation is seen at high and low temperatures (Koutrakis et al. 2005). Assuming that PM effect would not drop consistently as temperature increases, we added a cubic term in the model to allow for a plateau. We also examined whether the BRFSS factors modified $PM_{2.5}$ effects. The model is:

$$\hat{\beta}_{is} = \beta_0 + \beta_1 t_{is} + \beta_2 t_{is}^2 + \beta_3 t_{is}^3 + \beta_4 BRFSS_i \qquad [1.3]$$

where $\hat{\beta}_{is}$ is the estimated $PM_{2.5}$ coefficient for city *i* in season *s*, t_{is} is the centered temperature (i.e., temperature – mean temperature) for city *i* in season *s*, and $BRFSS_i$ is the BRFSS variable in city *i*. To estimate the effect of individual species, we performed the same meta-regression, but with the coefficient of the interaction term for species as the outcome being modeled. Here again we adjusted for city-season mean temperature as a surrogate for air exchange. We also investigated spatial variations between cities by focusing on a single outcome and exposure season to evaluate the effects in each city by mean exposure in that season for each city.

The effect estimates for $PM_{2.5}$ were expressed as the percent change in mortality associated with a 10 $\mu g/m^3$ increase in the 2-day averaged concentration of $PM_{2.5}$ mass, for comparability with most previous studies. We expressed the effect of species on mortality as the estimated percent increase in mortality at the 10^{th} and 90^{th} percentile of distribution of species-to-$PM_{2.5}$ proportion for each species, holding the $PM_{2.5}$ increase constant at 10 $\mu g/m^3$.

Data management was performed with SAS version 9.1 (SAS 2006), and regression analysis with R version 3.0.0 (R 2013).

Results

In this study, we examined 4,473,519 all-cause deaths, of which 1,429,968 were CVD, 308,235 MI, 255,430 stroke and 436,800 respiratory deaths.

Table 1-1 summarizes daily mortality, $PM_{2.5}$, temperature in all cities. On average, there were 28 non-accidental deaths per day. Daily death count by season was higher in the winter (n=31) and spring (n=28). Among the causes of interest, CVD killed the most people on average (9/day), followed by respiratory diseases (3/day). The overall mean concentration of $PM_{2.5}$ was 13.3 $\mu g/m^3$. $PM_{2.5}$ mean concentration was highest in the summer (15.0 $\mu g/m^3$) and lowest in the spring (11.6 $\mu g/m^3$). Some of the species exhibited strong seasonal variability. For example, sulfur varied from 798 ng/m^3 in the winter to 1669 ng/m^3 in the summer, with larger variations in some cities.

Table 1-1. Summary of daily mortality counts, $PM_{2.5}$, and temperature across all 75 cities in 2000–2006 (mean ± SD).

Variable	Overall	Spring	Summer	Fall	Winter
Mortality (no.)					
All causes	28.0 ± 33.9	28.2 ± 33.9	26.0 ± 31.4	27.1 ± 32.5	30.8 ± 37.2
CVD	9.0 ± 12.6	9.1 ± 12.6	8.2 ± 11.5	8.5 ± 11.8	10.0 ± 14.1
MI	1.9 ± 2.9	1.9 ± 2.9	1.8 ± 2.6	1.8 ± 2.7	2.2 ± 3.3
Stroke	1.6 ± 2.2	1.6 ± 2.2	1.5 ± 2.0	1.6 ± 2.1	1.8 ± 2.4
Respiratory diseases	2.7 ± 3.6	2.9 ± 3.7	2.3 ± 3.1	2.4 ± 3.2	3.3 ± 4.4
Temperature (˚C)	14.1 ± 10.0	13.4 ± 7.5	24.0 ± 4.1	15.1 ± 7.3	3.6 ± 8.0
$PM_{2.5}$ ($\mu g/m^3$)	13.3 ± 8.3	11.6 ± 6.5	15.0 ± 8.8	12.8 ± 8.4	13.9 ± 9.0
$PM_{2.5}$ species (ng/m^3)					
OC	4367 ± 2752	3688 ± 1806	4590 ± 2371	4491 ± 2716	4688 ± 3724
EC	724 ± 590	602 ± 438	628 ± 459	830 ± 647	842 ± 733
Na	80 ± 141	93 ± 165	89 ± 152	66 ± 117	71 ± 122
Al	31 ± 78	31 ± 55	51 ± 128	23 ± 45	15 ± 34
Si	117 ± 177	123 ± 134	171 ± 273	98 ± 125	69 ± 85
S	1174 ± 1019	1066 ± 731	1669 ± 1385	1107 ± 960	798 ± 512
K	79 ± 197	63 ± 49	103 ± 360	69 ± 62	79 ± 103
Ca	65 ± 77	65 ± 68	74 ± 77	68 ± 88	53 ± 72
V	2.5 ± 4.0	2.2 ± 3.4	2.7 ± 4.2	2.7 ± 4.4	2.5 ± 3.8
Fe	102 ± 124	93 ± 127	111 ± 111	108 ± 136	93 ± 121
Ni	2.5 ± 11.6	2.3 ± 6.0	2.2 ± 6.5	2.2 ± 5.7	3.2 ± 21.4
Cu	5.1 ± 8.9	4.2 ± 7.3	5.7 ± 11.5	5.0 ± 7.3	5.4 ± 8.6
Zn	18 ± 57	16 ± 39	16 ± 57	19 ± 53	22 ± 76

The distributions of monthly average proportions of $PM_{2.5}$ species are shown in Table 1-2. OC had the largest mean proportion (37.9%), followed by sulfur (8.78%) and EC (6.31%). The mean proportions for all the metals were less than 1% of mass concentration.

Table 1-2. Distributions of monthly species-to-$PM_{2.5}$ proportions (%) across all 75 cities (the 3-7th columns indicate 10th, 25th, …., 90th percentiles of the monthly species-to-$PM_{2.5}$ proportions).

Species	**Mean ± SD**	**10th**	**25th**	**50th**	**75th**	**90th**
OC	37.9 ± 16.9	24.6	29.1	35.5	44.1	53.6
EC	6.31 ± 3.45	2.86	3.96	5.51	7.49	10.1
Na	0.82 ± 1.31	0.07	0.20	0.45	0.96	1.90
Al	0.28 ± 0.44	0.03	0.07	0.15	0.31	0.64
Si	1.07 ± 1.22	0.30	0.45	0.70	1.20	2.12
S	8.78 ± 3.80	4.54	6.83	8.96	11.1	12.7
K	0.64 ± 0.52	0.31	0.40	0.53	0.72	0.98
Ca	0.62 ± 0.67	0.17	0.26	0.44	0.73	1.24
V	0.02 ± 0.03	0.00	0.01	0.01	0.03	0.05
Fe	0.89 ± 0.72	0.33	0.47	0.70	1.07	1.57
Ni	0.02 ± 0.06	0.00	0.00	0.01	0.02	0.04
Cu	0.04 ± 0.05	0.01	0.02	0.03	0.05	0.08
Zn	0.15 ± 0.18	0.04	0.06	0.10	0.15	0.24

Table 1-3 presents the estimated percent increase mortality for a 10 $\mu g/m^3$ increase in 2-day averaged $PM_{2.5}$ across the 75 cities. We found statistically significant associations between $PM_{2.5}$ and mortality. A 1.18% (95% CI: 0.93, 1.44%) increase in all-cause mortality was associated with a 10 $\mu g/m^3$ increase in the 2-day averaged concentration of $PM_{2.5}$. The greatest effect estimate effect was observed for stroke mortality [1.76% (95% CI: 1.01, 2.52%)], followed by respiratory deaths [1.71% (95% CI: 1.06, 2.35%)]. We observed seasonal variations in $PM_{2.5}$ effects. For a 10-$\mu g/m^3$ increase in 2-day averaged $PM_{2.5}$, the percent increases in all mortality categories were greatest in the spring.

Table 1-3. Estimated percent difference in mortality (95% CI) in association with a 10-µg/m3 increase in $PM_{2.5}$ at lag 0-1 by cause of death and season.

Mortality	Overall	Spring	Summer	Fall	Winter
All causes	1.18 (0.93, 1.44)	2.85 (2.23, 3.47)	0.85 (0.42, 1.28)	1.17 (0.72, 1.63)	0.46 (0.07, 0.85)
CVD	1.03 (0.65, 1.41)	2.47 (1.52, 3.43)	1.03 (0.38, 1.67)	0.87 (0.33, 1.42)	0.39 (-0.36, 1.14)
MI	1.22 (0.62, 1.82)	2.08 (0.72, 3.46)	1.23 (-0.19, 2.66)	0.81 (-0.32, 1.95)	0.41 (-1.12, 1.96)
Stroke	1.76 (1.01, 2.52)	3.31 (0.49, 6.22)	1.16 (-0.42, 2.76)	1.31 (0.05, 2.58)	1.59 (0.16, 3.03)
Respiratory diseases	1.71 (1.06, 2.35)	4.03 (2.85, 5.21)	1.09 (-0.58, 2.78)	0.58 (-0.39, 1.57)	0.86 (-0.11, 1.84)

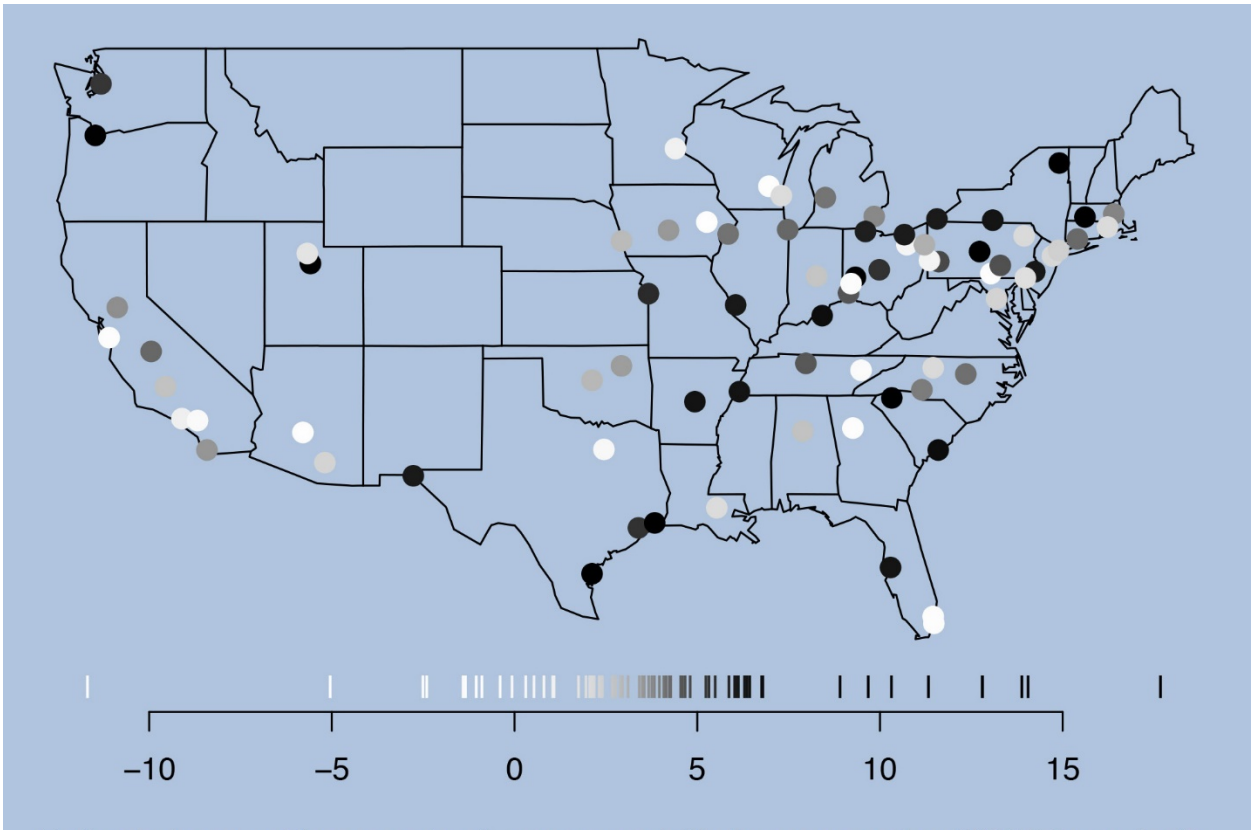

Figure 1-1. Spatial variations in estimated $PM_{2.5}$ effects between cities.

Figure 1-1 shows the effect estimates of $PM_{2.5}$ on all-cause mortality in each city by mean spring $PM_{2.5}$ in each city. We observed differential effects across cities.

Figure 1-2 shows the adjusted estimated percent increases in mortality for a 10-$\mu g/m^3$ increase in 2-day averaged $PM_{2.5}$ at the 10^{th} or 90^{th} percentile of distribution of the proportions of species. For all-cause mortality, interaction terms between $PM_{2.5}$ and species silicon, calcium, and sulfur had a *p*-value less than or equal to 0.1. We found that a 10-$\mu g/m^3$ increase in 2-day averaged $PM_{2.5}$ was associated with an increase in all-cause mortality of 3.55% (95% CI: 1.35, 5.81%)] at 90^{th} percentile of distribution of the sulfur-to-$PM_{2.5}$ proportion versus 2.16% (95% CI: 1.27, 3.06%) at the 10^{th} percentile of the sulfur-to-$PM_{2.5}$ ratio (data not shown). We also found silicon [3.25% (95% CI: 1.91, 4.62%) vs. 1.87% (95% CI: 1.42, 2.32%)] and calcium [3.42% (95% CI: 2.08, 4.77%) vs. 1.75% (95% CI: 1.34, 2.16%)] were associated with higher estimated effects of $PM_{2.5}$ on all-cause mortality. In addition, sulfur was associated with higher estimated $PM_{2.5}$ effect on respiratory deaths. The percent increase in respiratory mortality at the 90^{th} percentile of the sulfur-to-$PM_{2.5}$ proportion was 8.96% (95% CI: 1.55, 16.90%), vs. 4.44% (95% CI: 1.46, 7.51%) at the 10^{th} percentile.

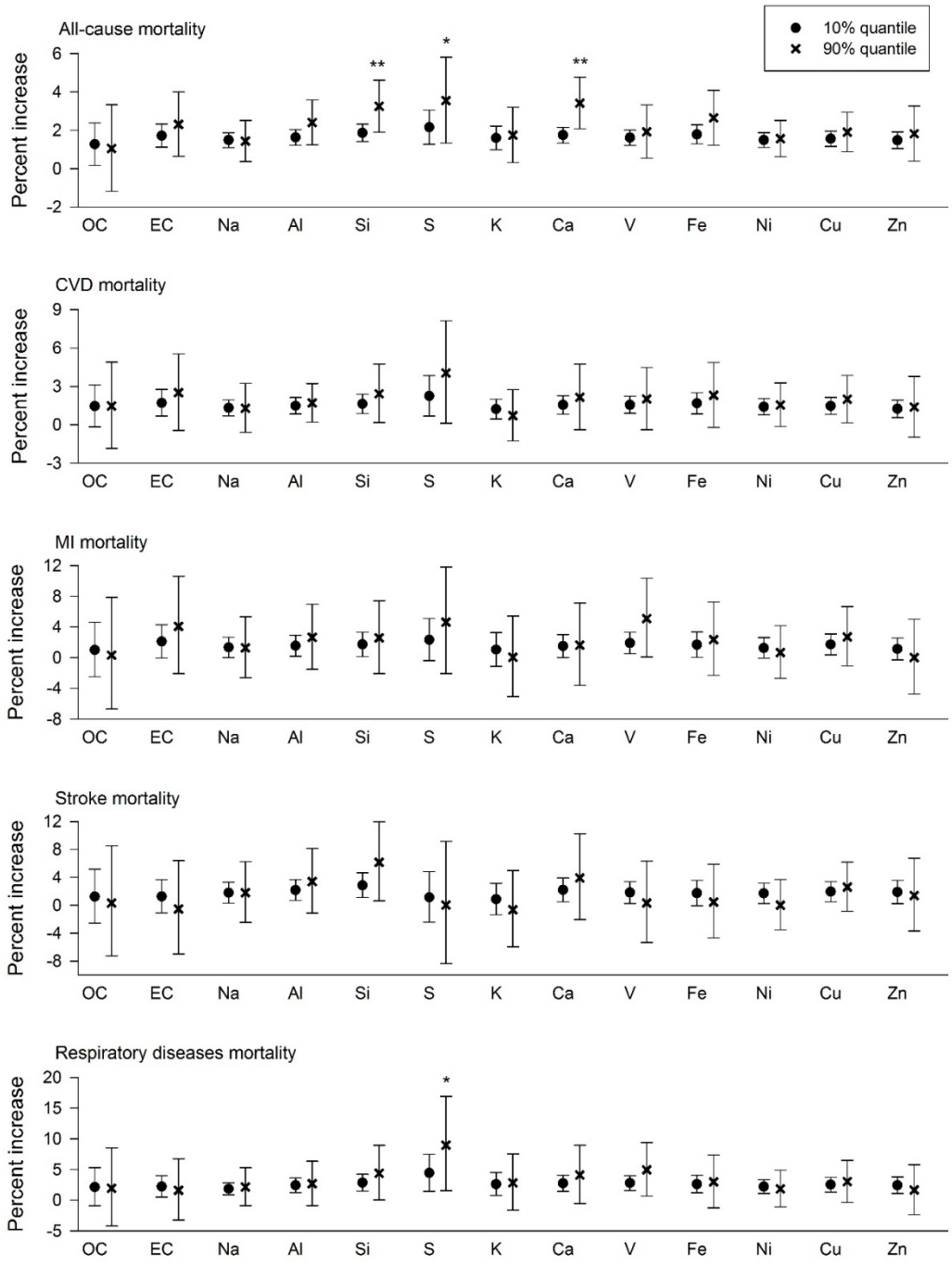

Figure 1-2. Estimated percent difference in mortality for a 10-μg/m3 increase in $PM_{2.5}$ at lag 0-1 and an increase of 10th or 90th percentile of distribution of monthly species-to-$PM_{2.5}$ proportions, controlled for city-season specific temperature (* indicates a p-value ≤ 0.1 for the interaction term, ** indicates a p-value ≤ 0.05 for the interaction term).

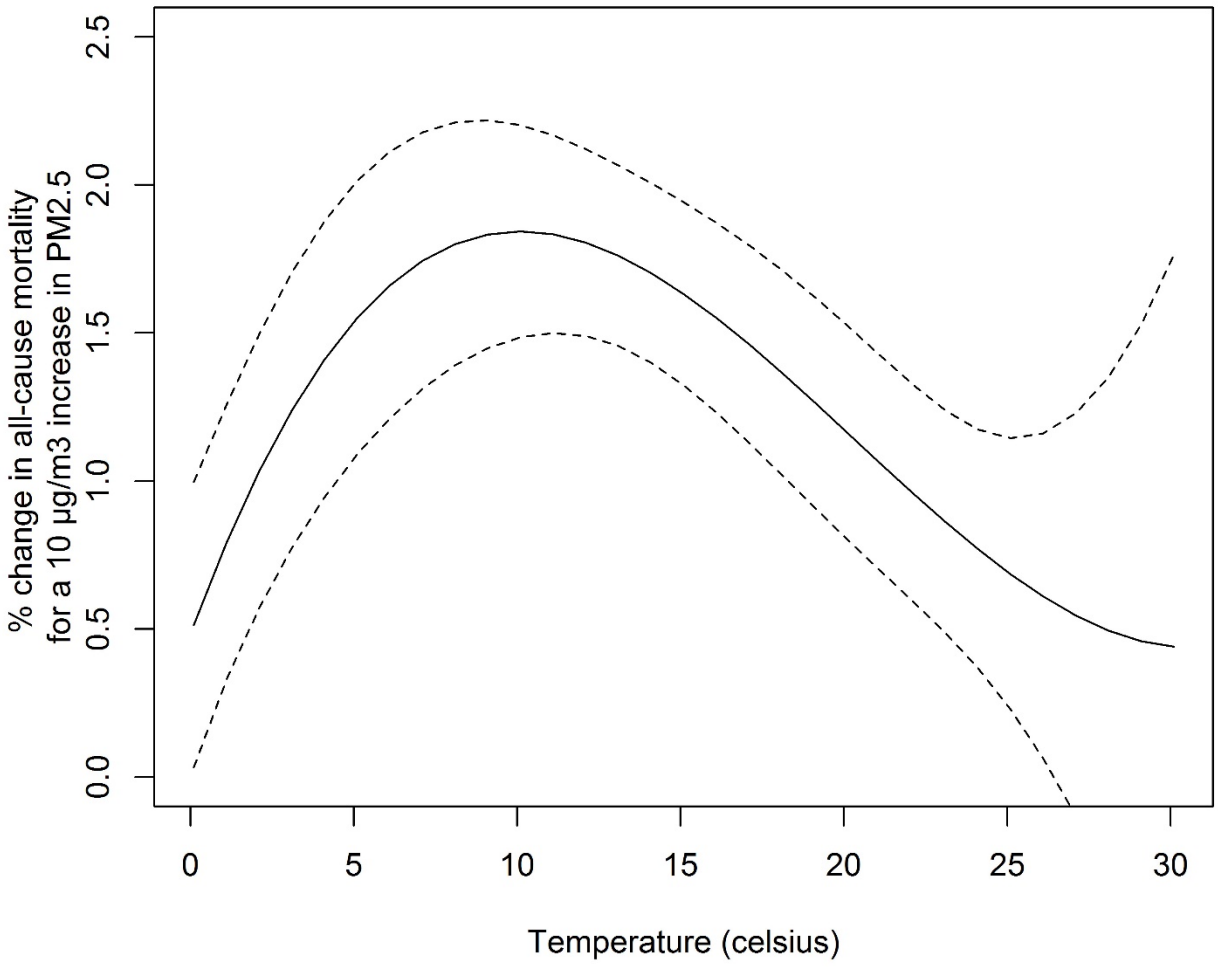

Figure 1-3. Relationship between estimated effects of $PM_{2.5}$ on all-cause mortality and temperature (controlled for smoking and alcohol consumption).

Figure 1-3 indicates the relationship between effect estimates and city-season temperature, which serves as a surrogate for ventilation and thus particle penetration indoors. We observed an inverted U-shape relationship with a plateau at high temperatures. The *p*-value for cubic term is 0.06 in meta-regression without BRFSS factors and is 0.07 controlled for smoking and alcohol

consumption. The all-cause mortality effect estimates first increase as temperature increases and peak around a seasonal average of 10 ˚C. After that, they decrease until they reach a plateau at around 28 ˚C.

County-level percent of green space, diabetes, obesity, asthma, or physical activity did not modify the effect of $PM_{2.5}$ on mortality. However, among the behavioral factors, we found the effects of $PM_{2.5}$ were higher in areas where people smoked more or had two drinks or more per day. Specifically, an IQR increase in the prevalence of smokers (8.8%) was associated with a 34% increase in estimated $PM_{2.5}$ effects, while an IQR increase in the prevalence of heavy drinkers (7.0%) was associated with an increase of 40% in the estimated effects of fine particles.

Discussion

In this nationwide time-series study, we estimated the effects of $PM_{2.5}$ mass and species on daily mortality across 75 U.S. cities, covering over 4 million deaths. We found an increase in $PM_{2.5}$ concentration at lag day 0-1 was statistically significantly associated with increased risk of all-cause mortality, CVD, MI, stroke, and respiratory mortality. We also found that $PM_{2.5}$-related effects were modified by certain species. Furthermore, analysis by season indicated that effect estimates were highest in the spring. To investigate this seasonal pattern we included city-season specific temperature in the meta-regression analysis. These seasonal variations may affect the characteristics of $PM_{2.5}$ mixture and mediate its effects on health outcomes (Bell et al. 2007).

Controlling for this potential confounder and for $PM_{2.5}$ mass, we found that a species related to coal combustion (i.e., sulfur) was associated with higher risks for all cause but particularly

respiratory mortality. Sulfur is also a marker of regional pollution thus it may not only reflect exposures to power plant emissions. Changes in the proportion of OC mass in $PM_{2.5}$ did not modify its effect on mortality for any cause, suggesting this species has average toxicity. We found higher silicon or calcium proportions were associated with increased estimated $PM_{2.5}$ mortality risks. These crustal elements are often elevated near roads and can be a surrogate for increased road dust, which in addition to those elements contains various organic compounds, compounds from tire and brake wear, etc. (Rogge et al. 1993). Thus they may be a marker for pollution from traffic other than EC.

The BRFSS factors we examined were on the county level. The distributions of prevalence of smoking and heavy drinkers (i.e., > two drinks/day) in different cities were approximately normal distributed with a mean around 30% and 60%, respectively. We found that cities with more smokers or heavy drinkers had larger estimated effects of $PM_{2.5}$. These have not previously been identified as susceptibility factors for the effects of particles on health, and this requires greater attention.

The magnitudes of effects in our study are comparable to those reported by other studies. For example, a study that included 112 U.S. cities reported a 0.98% (95% CI: 0.75, 1.22%) increase, a 0.85% (95% CI: 0.46, 1.24%) increase, a 1.18% (95% CI: 0.48, 1.89%) increase, a 1.78% (95% CI: 0.96. 2.62%) increase, and a 1.68% (95% CI: 1.04, 2.33%) increase in all-cause, CVD, MI, stroke, and respiratory mortality, respectively, for a 10-$\mu g/m^3$ increase in 2-day averaged $PM_{2.5}$ (Zanobetti and Schwartz 2009). Our estimates are slightly higher than the above ones and are closer to those by a 27-city study, which found a 1.21% (95% CI: 0.29, 2.14%) increase in all-cause mortality, a 1.78% (95% CI: 0.20, 3.36%) increase in respiratory mortality and 1.03% (95% CI: 0.02, 2.04%) increase in stroke mortality for a 10 $\mu g/m^3$ increase in previous day's $PM_{2.5}$

(Franklin et al. 2007). Our study and these two studies used city-season specific models to allow for seasonal differences in the effects of temperature and day of the week.

The finding that effects were highest in the spring is consistent with previous studies (Zanobetti and Schwartz 2009; Zeka et al. 2006). Franklin et al. (2008) found similar pattern using linear and quadratic temperature in the meta-regression. Additionally, we included the cubic term, which was marginally significant and led to the small plateau at high temperatures. These results indicated greater effects for moderate temperatures when windows are more likely to be open and particle penetration rates are higher.

EC is considered as a marker of traffic emissions (Viana et al. 2006). Previous research has reported EC was significantly associated with increased risk of mortality due to all causes or cardiovascular diseases (Bell et al. 2009; Metzger et al. 2004; Peng et al. 2009). In this study, we did observe that increase in the EC-to-$PM_{2.5}$ proportion increased the association between $PM_{2.5}$ and all-cause mortality and CVD mortality in crude meta-regression, but it was no longer significant when we controlled for city-season temperature. Similarly, there were two studies that also controlled for temperature in the meta-regression and did not find any effect modification by EC in the association between $PM_{2.5}$ and non-accidental mortality or hospital admissions for cardiovascular diseases (Franklin et al. 2008; Zanobetti et al. 2009).

Silicon and calcium, which may be associated with soil or road dust, were observed to modify the effects of $PM_{2.5}$ on all-cause mortality in our study. Crustal elements have been reported to have adverse effects on health. For example, Ostro et al. found strong association between silicon and mortality (Ostro et al. 2010); Franklin et al. (2008) observed silicon and aluminum were modifiers of the $PM_{2.5}$-mortality effects. There were also studies that showed plausible biological

mechanisms of inflammatory effects of road dust containing aluminum and/or silicon (Becker et al. 2005; Clarke et al. 2000). Additionally, road dust is often coated with organic compounds and metals from car exhaust, tire wear, etc. (Rogge et al. 1993), that may contribute to its toxicity.

Nickel, as a marker of oil combustion, was reported to have effect modification in the relationship between $PM_{2.5}$ and mortality or hospital admissions in previous studies (Franklin et al. 2008; Zanobetti et al. 2009), but we did not observe any. On average, nickel only accounted for 0.02% of the $PM_{2.5}$ concentration in this study. The concentrations of nickel are frequently lower than the method detection limit (Burnett et al. 2000), which may make us fail to detect its effects. Nevertheless, toxicological research has found evidence on its adverse effects (Gao et al. 2004; Lippmann et al. 2006). For example, Lippmann et al. (2006) found atherosclerotic prone mice that were exposed to concentrated air particles had a pronounced acute change in heart rate and heart rate variability when nickel was especially high. Lippmann and the New York studies which found nickel effects had high exposures due to the residual fuel burn in New York for heating. The levels for the entire country are lower.

We observed the effect of $PM_{2.5}$ mass on all-cause and respiratory mortality was modified by sulfur. This component is a marker of coal combustion emissions, which suggests species derived from coal combustion might have great toxicity on mortality, particularly due to respiratory diseases. Sulfate is the primary form of sulfur in particles. Sulfate has been implicated as a major toxic species in $PM_{2.5}$ (Amdur 1996) and reported to be associated with increased risk of various mortality outcomes in earlier epidemiological studies (Fairley 1999; Hoek et al. 2000; Laden et al. 2000; Mar et al. 2000). The importance of sulfates in the air may be due to the ability of acid sulfates to solubilize transition metals and thus making them bio-available (Ghio et al. 1999). There were studies that found sulfate was associated with endothelial dysfunction (O' Neill et al.

2005), increased oxidative stress and coagulation (Chuang et al. 2007). These toxicology findings provide plausibility to sulfate health effects.

One disappointing aspect of this result is that despite the use of 75 cities and almost 4.5 million deaths, we were unable to distinguish much difference in toxicity for many of the species we examined. This may reflect only modest differences in toxicity, but may also reflect more fundamental difficulties in identifying differences between many correlated exposures with limited measurements over time. Evidence of the low power to detect differences can be seen in the difference between our results for all deaths and results for cardiovascular deaths. The pattern of higher estimated effects when PM mass has a larger fraction of silicon, sulfur, and calcium is present for cardiovascular deaths as well, but with a third as many deaths, it does not reach significance. One option to improve study power might be specifically selecting locations with high proportions of the species of interest.

There are several limitations in this study. First, our ability to capture spatial variability is constrained due to the location of U.S. EPA monitors. A previous study showed moderate to low monitor-to-monitor correlations between daily concentrations of several species (arsenic, EC, and nickel) in the New York City area, which suggest high spatial variability in some speciation concentrations (Ito et al. 2004). Differential measurement error between species that are better or worse represented by a single monitor may bias differential results. However, in a time-series study much of the geographic variability will result in Berkson error (Zeger et al. 2000), which will not produce bias. Meanwhile, failing to capture spatial variability might weaken study power and attenuate estimates. Second, we failed to capture day-to-day variation in the analysis. Although we used monthly average species-to-$PM_{2.5}$ proportions to gain more power, we still lost variation across days. Nevertheless, we believe that the day-to-day variation is random error in

measurement, which induces little or no bias. Third, as mentioned above, there are data limitations, such as the one-in-six and one-in-three sampling frequency for the species. Whether one takes the approach of Krall et al. (2013) and only analyzes those days, or our approach and gains power by analyzing every day but with more error prone monthly means of the species, there is a price that is paid for this lack of data. Together with moderate correlation among the species and with total particle mass, this makes the task difficult. We do not believe this is likely to produce false positives, and hence we believe our findings are well supported.

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Study 2. Use of the Adaptive LASSO Method to Identify PM2.5 Components Associated with Blood Pressure in Elderly Men: The Veterans Affairs Normative Aging Study

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Abstract

Background: $PM_{2.5}$ has been associated with adverse cardiovascular outcomes, but it is unclear whether specific $PM_{2.5}$ components, particularly metals, may be responsible for cardiovascular effects.

Objectives: to determine which $PM_{2.5}$ components are associated with blood pressure in a longitudinal cohort.

Methods: We fit linear mixed-effects models with the adaptive LASSO penalty to longitudinal data from 718 elderly men in the Veterans Affairs Normative Aging Study, 1999-2010. We controlled for $PM_{2.5}$ mass, age, body mass index, use of antihypertensive medication (ACE inhibitors, non-ophthalmic beta blockers, calcium channel blockers, diuretics, and angiotensin

receptor antagonists), smoking status, alcohol intake, years of education, temperature, and season as fixed effects in the models, and additionally applied the adaptive LASSO method to select $PM_{2.5}$ components associated with blood pressure. Final models were identified by the Bayesian Information Criterion (BIC).

Results: For systolic blood pressure (SBP), Ni and Na were selected by the adaptive LASSO, whereas only Ni was selected for diastolic blood pressure (DBP). An IQR increase (2.5 ng/m^3) in 7-day moving average Ni was associated with 2.48 (95% CI: 1.45, 3.50) mm Hg increase in SBP and 2.22 (95% CI: 1.69, 2.75) mm Hg increase in DBP, respectively. Associations were comparable when the analysis was restricted to study visits with $PM_{2.5}$ below the 75^{th} percentile of the distribution (12 $\mu g/m^3$).

Conclusions: Our study suggested exposure to ambient Ni was associated with increased blood pressure independent of $PM_{2.5}$ mass in our study population of elderly men. Further research is needed to confirm our findings, assess generalizability to other populations, and identify potential mechanisms for Ni effects.

Introduction

Studies have shown that exposure to fine particulate matter ($PM_{2.5}$, particles $\leq$ 2.5 μm in aerodynamic diameter) is associated with cardiovascular morbidity and mortality (Franklin et al. 2008; Laden et al. 2006; Miller et al. 2007; Zanobetti et al. 2009). $PM_{2.5}$ consists of various components, including organic and elemental carbon, metals, and ions. Some national studies have evaluated whether $PM_{2.5}$ components may have differential effects on cardiovascular health (Dai

et al. 2014; Peng et al. 2009), but it still is not clear whether specific components may be responsible for $PM_{2.5}$-related cardiovascular effects.

Increased blood pressure is a major risk factor for cardiovascular events. Several studies have investigated the relationship between PM and blood pressure. However, the results have varied, possibly because of differences in the particle composition (Baccarelli et al. 2011; Choi et al. 2007; Chuang et al. 2010; Dvonch et al. 2009; Harrabi et al. 2006; Hoffmann et al. 2012; Ibald-Mulli et al. 2004; McCracken et al. 2007; Schwartz et al. 2012; Wilker et al. 2009; Wilker et al. 2010).

Inhaled PM-associated metals may be able to translocate from lung into systemic circulation and induce adverse effects on cardiovascular system (Wallenborn et al. 2007). There is growing evidence supporting adverse effects of ambient metals on cardiovascular health. For example, Fe, K, Ti, and Zn in fine particles were positively associated with cardiovascular mortality in a California study (Ostro et al. 2007). A multiple-community study reported that Ni and Na^+ modified associations of $PM_{2.5}$ on hospital admissions due to cardiovascular diseases (Zanobetti et al. 2009). There were also numerous animal studies that reported cardiovascular toxicity of PM metal components Zn, Ni, and V (Campen et al. 2001; Chuang et al. 2013; Kodavanti et al. 2008; Lippmann et al. 2006). In terms of sources, PM-associated metals usually come from road dust (e.g., Ca, Al, Fe, and Ti), oil combustion (e.g., Ni and V), traffic emission (e.g., Zn and Cu), wood burning (e.g., K), and sea salt (e.g., Na).

In this study, we examined the association between blood pressure and 11 $PM_{2.5}$ components, including 8 metals (Fe, K, Al, Ni, V, Cu, Zn, and Na) and 3 non-metals (S, Si, and Se), with longitudinal data from the Veterans Affairs Normative Aging Study.

Methods

Study population

The Normative Aging Study (NAS) was established in 1963 by the Department of Veterans Affairs (Bell et al. 1972). Briefly, it is an ongoing longitudinal study of aging, which enrolled 2,280 community-dwelling, healthy men living in the Greater Boston area. Participants were free of known chronic medical conditions at enrollment and have undergone examinations every 3 to 5 years, including physical examinations and questionnaires. All participants provided written informed consent. The study was reviewed and approved by the Institutional Review Boards of all participating institutions.

After excluding participants with incomplete information for any of the covariates of interest, those who died, or those who moved out of New England, a total of 718 participants with 1,567 observations had examinations between March 1999 and October 2010. Of the 718 participants, 235 (33%) had one visit, 195 (27%) had two visits, and 288 (40%) had three or more visits.

Blood pressure measurements

During a clinical visit, a physician uses a standard mercury sphygmomanometer with a 14-cm cuff to measure blood pressure for the subject while he is sitting, including systolic blood pressure (SBP) and fifth-phase diastolic blood pressure (DBP) in each arm to the nearest 2 mm Hg. We used the means of the left and right arm measurements as a subject's SBP and DBP.

Environmental data

Daily ambient $PM_{2.5}$ and its components were measured at the stationary ambient monitoring site at the Harvard University Countway Library (Kang et al. 2010), using the Tapered Element Oscillating Microbalance (TEOM 1400a, Rupprecht & Patashnick Co.) and the Energy Dispersive X-ray Fluorescence Spectrometer (Epsilon 5, PANalytical), respectively. The monitoring site is 1 km from the clinical examination site. We obtained daily temperature data from Boston Logan airport weather station.

Statistical analysis

We used 7-day moving average concentrations for $PM_{2.5}$ and the following 11 components, K, S, Se, Al, Si, Fe, Ni, V, Cu, Zn, and Na, since previous studies have suggested that PM averaging over that time period is strongly associated with blood pressure (Mordukhovich et al. 2009; Wilker et al. 2010; Zanobetti et al. 2004). We focused on these components because their concentration levels are mostly above the method detection limits and they are representative of different PM sources (Hopke et al. 2006). In the analysis, we controlled for continuous variables age, body mass index [BMI, computed as weight (in kilograms) divided by height (in square meters)], years of education, linear and quadratic terms of mean temperature of visit day, and categorical variables use of each individual types of antihypertensive medication (ACE inhibitors, non-ophthalmic beta blockers, calcium channel blockers, diuretics, and angiotensin receptor antagonists), smoking status (3 categories: never, former, current smoker), alcohol intake (whether the participant takes two or more drinks per day, yes or no), and season (4 categories; defined as spring: March-May, summer: June-August, fall: September-November, winter: December-February) regardless of

statistical significance as these variables have been shown to predict cardiovascular health (Mordukhovich et al. 2009; Schwartz et al. 2012). In addition, we adjusted for potential confounding of associations with $PM_{2.5}$ components by $PM_{2.5}$ mass (Mostofsky et al. 2012). All variables were measured at each visit. We forced these covariates to be included in the models and estimated their fixed effects with no penalization.

Selecting important predictors from a large list of correlated predictors is difficult, and most methods are empirical. Approaches such as stepwise methods ignore stochastic errors inherited in the stages of variable selection (Fan and Li 2001) and can yield falsely narrow confidence intervals (Harrell 2001). To improve on this, we applied the adaptive LASSO method to select important component(s) that may be associated with blood pressure from those 11 $PM_{2.5}$ components. Briefly, the LASSO (Least Absolute Shrinkage and Selection Operator) is a regression shrinkage and selection approach that applies an ℓ_1 penalty to the component regression coefficients. This penalty essentially minimizes the sum of squared errors subject to the sum of the absolute values of the coefficients being less than a given value (Tibshirani 1996). The adaptive LASSO is a later version of the LASSO, which uses weights for penalizing different coefficients in the ℓ_1 penalty and enjoys the oracle properties, which means, given that the true model depends only on a subset of the predictors, this selection procedure is able to identify the right subset model and satisfies asymptotic normality (Fan and Li 2001; Zou 2006). Since subjects had repeated measures, we fit linear mixed-effects models with random subject-specific intercepts to capture the correlation among different measurements within the same subject, as follows:

$$Y_i=\boldsymbol{X}_i\boldsymbol{\alpha}+\boldsymbol{Z}_i\boldsymbol{\beta}+\mu_i+\varepsilon_i\,, \qquad [2.1]$$

where, Y_i is the blood pressure level (SBP or DBP) of subject i, $\boldsymbol{X}_i = (X_{i1}, \ldots, X_{iP})^T$ is a vector of $PM_{2.5}$ mass and other covariates, $\boldsymbol{Z}_i = (Z_{1i}, \ldots, Z_{iM})^T$ is a vector of $PM_{2.5}$ components, μ_i is the random intercept. Hence, $\boldsymbol{\alpha}$ indicates the fixed effects of $PM_{2.5}$ mass and other covariates $\boldsymbol{X}_i$, and $\boldsymbol{\beta}$ is the penalized effects of $PM_{2.5}$ components $\boldsymbol{Z}_i$ that are given by the adaptive LASSO.

First, we used the ordinary linear mixed-effects (LME) model to obtain non-zero coefficients (β_{lme}) for each component, and computed the adaptive weight as its inverse ($w = 1/\beta_{lme}$). Heuristically, this allows us to give less weight in the penalty to variables whose standardized regression coefficients are large, since they are more likely to be predictors. When using the adaptive LASSO, we assign a non-negative penalty parameter, λ, to determine how strongly we penalize, or restrict, the magnitude of the $PM_{2.5}$ components regression coefficients. When λ is equal to 0, there is no shrinkage and the model is just the ordinary mixed-effects regression of the fixed covariates and all components; when it is large enough, there is maximum shrinkage, yielding a model that includes fixed covariates only and all component coefficients equal to 0; when λ takes some value in between, some coefficients are 0, and the model is a penalized model. Components with non-zero coefficients are "selected" by the adaptive LASSO. In this way, the method chooses $PM_{2.5}$ components that may be associated with the outcomes. We ran the models across that range of λ's, i.e., from no shrinkage to maximum shrinkage, and chose the λ having the smallest Bayesian Information Criterion (BIC) (Schwarz 1978). Last, we used the mixed-effects model with fixed covariates and selected components only to obtain the estimated effects and corresponding 95% confidence intervals.

In a sensitivity analysis, we omitted study visits with $PM_{2.5}$ below the 75th percentile of the distribution (12 μg/m^3).

Data cleaning was performed with SAS 9.3 (SAS Institute Inc.), and data analysis was performed with R 3.0.1 (http://www.r-project.org/).

Results

Table 2-1 summarizes the characteristics of study population. Subjects in this study were elderly men, with a mean age of 73 years (SD = 7 years) at the first visit. Average SBP and DBP at the first visit were 132 mm Hg (SD = 17 mm Hg) and 76 mm Hg (SD = 10 mm Hg), respectively.

Table 2-1. Characteristics of subjects in the study.

Variable	**First visit (n=718)**	**All visits (n=1,567)**
Mean ± SD		
SBP (mm Hg)	131.6 ± 16.7	128.1 ± 17.6
DBP (mm Hg)	75.9 ± 9.9	71.9 ± 10.3
Age (years)	72.8 ± 6.8	74.7 ± 6.8
BMI (kg/m^2)	28.2 ± 4.0	28.0 ± 4.1
Education (years)	14.6 ± 2.8	14.6 ± 2.8
Number (%)		
Use of ACE inhibitors	197 (27%)	540 (34%)
Use of non-ophthalmic beta blockers	213 (30%)	554 (35%)
Use of calcium channel blockers	104 (14%)	265 (17%)
Use of diuretics	150 (21%)	381 (24%)
Use of angiotensin receptor antagonists	36 (5%)	124 (8%)
Current smokers	28 (4%)	47 (3%)
Former smokers	488 (68%)	1049 (67%)
Two or more drinks per day	143 (20%)	299 (19%)

$PM_{2.5}$ and component concentrations are shown in Table 2-2. 7-day moving average $PM_{2.5}$ across all study visits had a mean of 10 $\mu g/m^3$ (SD = 3.7 $\mu g/m^3$), with an IQR of 4.3 $\mu g/m^3$. S accounted for the largest proportion of the total $PM_{2.5}$ concentration (10.4%), followed by Na (1.9%). The average concentration of Ni was 3.1 ng/m^3 (SD = 2.5 ng/m^3), and it only accounted for 0.03% of the mass concentration.

Table 2-2. Mean $PM_{2.5}$ mass and component concentrations across all study visits.

Pollutant	**Mean ± SD**	**IQR**	**Proportion of $PM_{2.5}$ (%)**
$PM_{2.5}$ ($\mu g/m^3$)	10.0 ± 3.7	4.3	
Components (ng/m^3)			
Fe	68.1 ± 24.2	21.5	0.7
K	39.2 ± 24.6	16.9	0.4
S	1039.1 ± 513.2	554.1	10.4
Al	51.8 ± 27.8	21.1	0.5
Si	76.7 ± 51.1	38.4	0.8
Ni	3.1 ± 2.5	2.5	0.03
V	3.5 ± 2.3	2.6	0.04
Cu	3.5 ± 1.2	1.5	0.04
Zn	11.4 ± 6.0	5.8	0.1
Se	0.2 ± 0.3	0.3	0.002
Na	190.7 ± 72.4	92.8	1.9

Figure 2-1 shows the relationship between BIC, a criterion for model selection and λ, the adaptive LASSO penalty parameter. For SBP models, the model with the smallest BIC had λ = 4 and Ni and Na as the only two among the eleven $PM_{2.5}$ components (i.e., K, S, Se, Al, Si, Fe, Ni, V, Cu, Zn, and Na) with non-zero coefficients, whereas all component coefficients were zero when λ = 9. For DBP models, the model with the smallest BIC had λ = 13 and Ni as the only component with a non-zero coefficient, whereas all component coefficients were zero when λ = 30.

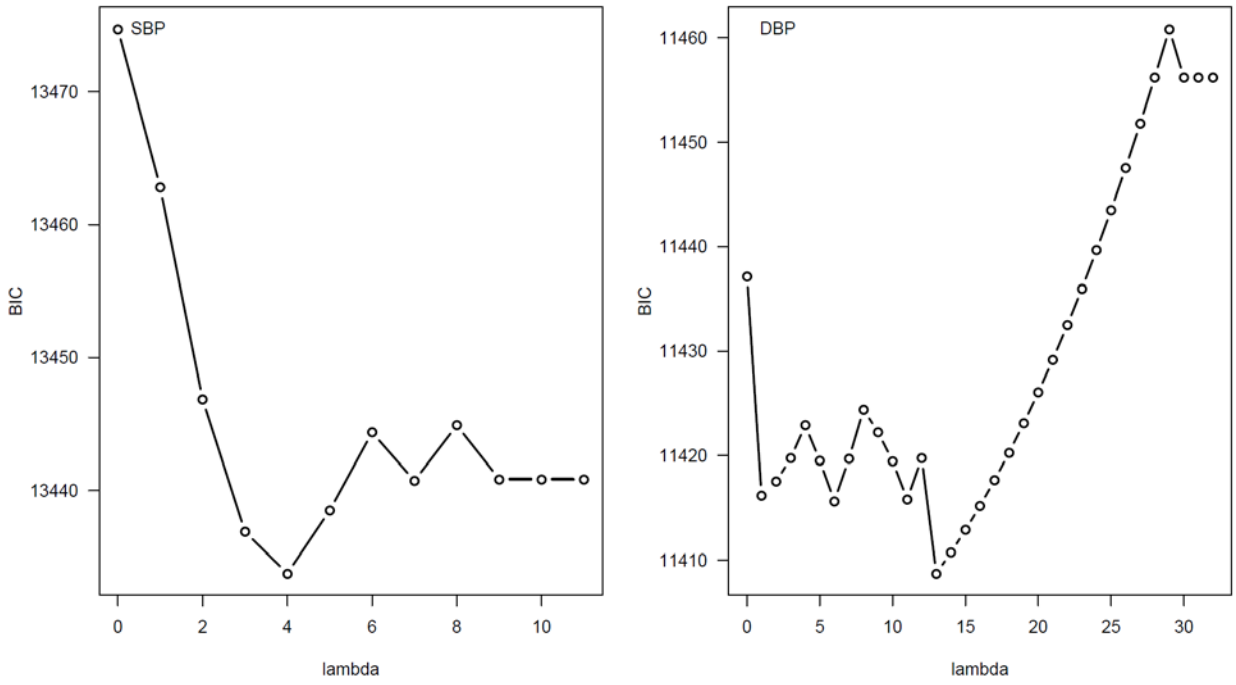

Figure 2-1. The relationship between BIC, a criterion for model selection and λ, the adaptive LASSO penalty parameter.

In models fitted using only the selected components, we found an IQR increase (2.5 ng/m^3) in 7-day moving average Ni was associated with a 2.48 (95% CI: 1.45, 3.50) mm Hg increase in SBP and 2.22 (95% CI: 1.69, 2.75) mm Hg increase in DBP, respectively. To compare with other studies, we also estimated the effects of $PM_{2.5}$ mass: every 10 μg/m^3 increase in 7-day moving average $PM_{2.5}$ was associated with 1.36 (95% CI: -1.67, 4.39) mm Hg increase in SBP and 0.61 (95% CI: -0.85, 2.07) mm Hg increase in DBP, respectively.

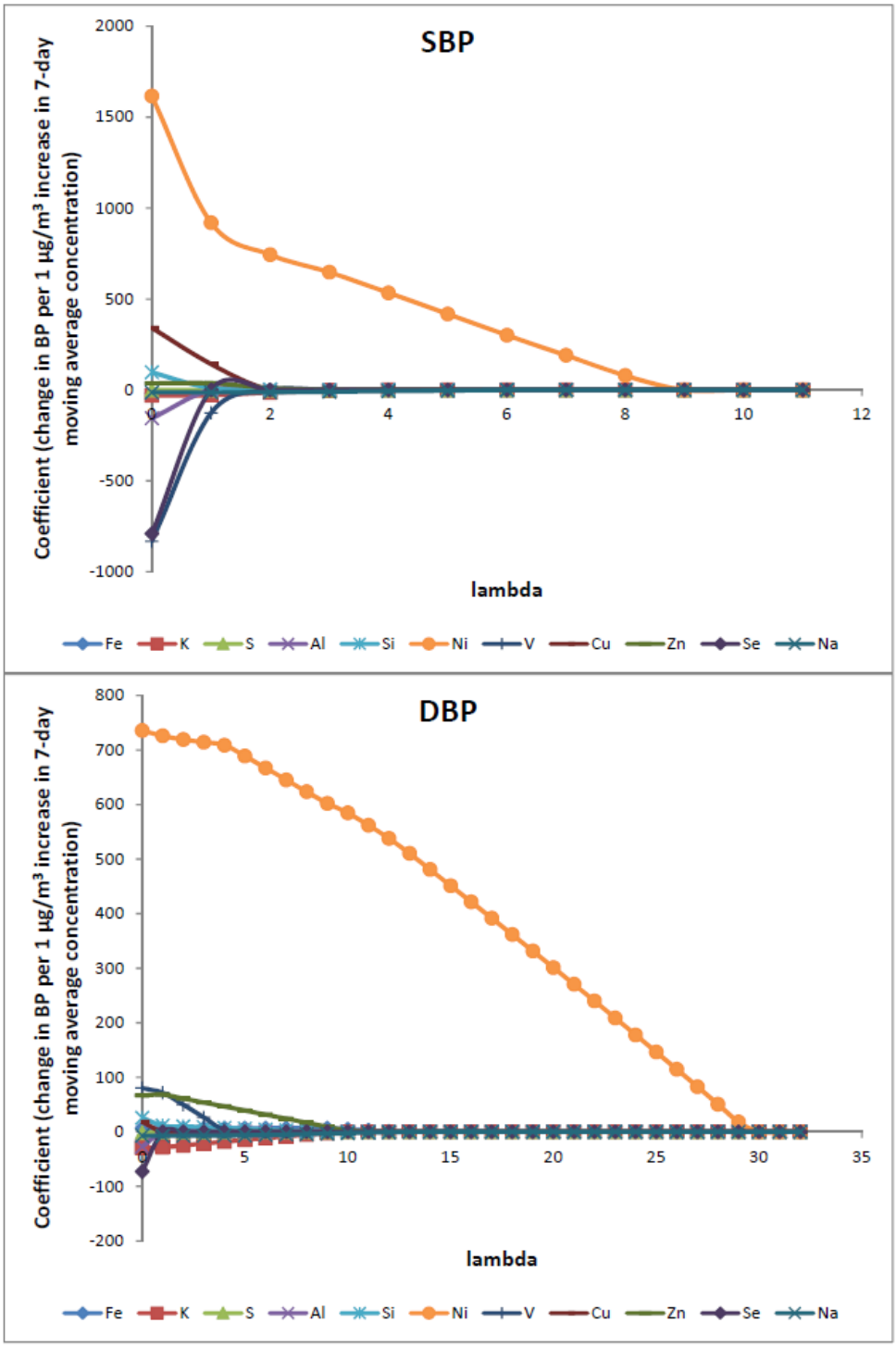

Figure 2-2. LASSO coefficient paths: plot of coefficient profiles for $PM_{2.5}$ components as a function of λ.

LASSO coefficient paths for SBP and DBP are shown in Figure 2-2. Each component coefficient is expressed as the change in mean SBP or DBP per 1-μg/m^3 increase in the 7-day moving average concentration of the $PM_{2.5}$ component. Each curve indicates the rate at which the component coefficient shrinks towards zero as λ increases. When λ = 0, all components have non-zero coefficients.

Table 2-3. Comparison of estimated coefficients of Ni in the main analysis and in the sensitivity analysis where study visits with 7-day moving average $PM_{2.5} \geq 12$ μg/m3 were excluded.

Analysis (No. of visits)	SBP Coefficient	*p*-value	DBP Coefficient	*p*-value
Main analysis (N = 1,567)	0.989	< 0.001	0.888	< 0.001
Sensitivity analysis (N = 1,201)	1.149	< 0.001	1.104	< 0.001

Table 2-3 shows the comparison of results from the main analysis and the sensitivity analysis that was restricted to data from study visits with $PM_{2.5}$ concentrations below the 75th percentile of the distribution (12 μg/m^3). We found that the estimated coefficients of Ni for both SBP and DBP in the sensitivity analysis were comparable to those in the main analysis, and their statistical significance remained. That is, Ni was associated with SBP and DBP even when overall $PM_{2.5}$ concentrations were restricted to < 12 μg/m^3.

Discussion

In this study, we used the adaptive LASSO shrinkage method to choose $PM_{2.5}$ components that might be related to blood pressure in a cohort of elderly men. We found that 7-day moving average Ni and Na were associated with SBP, and 7-day moving average Ni concentration was also associated with DBP. This association persisted when restricted to data from study visits with $PM_{2.5}$ concentrations below 12 $\mu g/m^3$.

Ni in ambient air is considered a marker of oil combustion; other sources of Ni include coal combustion, nickel metal refining, sewage sludge incineration, and manufacturing facilities. (EPA 2000). There have been a number of toxicological studies that examined the effects of ambient Ni on cardiovascular health. In a mouse model of atherosclerosis, mice had acute changes in heart rate and heart rate variability when exposed to concentrated fine PM (average concentration of Ni was 43 ng/m^3 and there were Ni peaks at ~ 175 ng/m^3) (Lippmann et al. 2006). Another animal study showed that Ni inhalation caused a decrease of 75 bpm in maximal heart rate at the concentration of 1.3 mg/m^3 and a decrease of 100 bpm at 2.1 mg/m^3 in rats (Campen et al. 2001). Moreover, Ni was reported to induce increases in pulmonary protein leakage and perivascular and peribronchiolar inflammation in both normotensive and spontaneously hypertensive rats that were intratracheal instilled with 1.5 μmol/kg of $NiSO_4 \bullet 6H_2O$ in saline (Kodavanti et al. 2001). A similar study found alterations in heart rate variability (HRV) related to PM exposure were Ni-dependent in spontaneously hypertensive rats after adjusted for HRV responses in control rats (Chuang et al. 2013).

Several epidemiological studies have provided evidence of cardiovascular effects of Ni. A national study conducted in 106 U.S. counties reported that associations between $PM_{2.5}$ concentrations and

cardiovascular and respiratory hospitalizations were stronger when Ni was high (Bell et al. 2009). Zanobetti et al. (2009) examined associations of $PM_{2.5}$ with emergency hospital admissions in 26 U.S. communities and found that Ni significantly modified the association between $PM_{2.5}$ mass and hospital admissions for cardiac diseases and myocardial infarctions. A recent study found a significant association between ischemic heart diseases mortality and Ni based on data from the American Cancer Society (Lippmann et al. 2013). On the other hand, Zhou and co-authors (2011) failed to find cumulative effects from lag 0 to lag 2 of Ni in Detroit or Seattle. A more recent nationwide study that included 75 U.S. cities did not observe any effect modification of Ni in the $PM_{2.5}$-mortality association (Dai et al. 2014).

There are several possible reasons for the differences in these epidemiological studies. First, Ni concentrations are usually lower than the method detection limits, which makes it difficult to determine whether associations are present (Burnett et al. 2000). New York counties had particularly high levels of Ni (a mean of 19.0 ng/m^3 Ni in New York fine PM vs. a mean of 1.9 ng/m^3 Ni in national fine PM) due to combustion of residual oil-fired power plants and ocean-going ships (Lippmann et al. 2006). In a reanalysis of the National Morbidity, Mortality, and Air Pollution Study (NMMAPS) data, Dominici et al. (2007) found evidence of effect modification by Ni, which was consistent with Lippmann et al. (2006); however, the effect modification of Ni on PM-mortality association was much weaker and no longer statistically significant when New York counties were excluded from the analysis. In the two studies that did not find significant associations of Ni, Ni had a relatively low level. For example, the national mean concentration of Ni was 2.5 ng/m^3 in Dai et al. (2014). Given the substantial differences in Ni concentrations, it is conceivable that studies conducted in other places or nationally may not be able to observe the health effects of Ni as the New York studies did. In our study, Ni had an average concentration of

3.1 ng/m^3, which was higher than the national mean but still much lower than New York levels (Dominici et al. 2007; Lippmann et al. 2006). Furthermore, it is possible that Ni interacts with other PM components to pose an increased risk to health. Campen et al. (2001) reported evidence of a synergistic interaction between Ni and V, both of which are markers of PM from oil combustion. Hence, the heterogeneous composition of PM in different locations might lead to different estimated effects of Ni.

Na also was selected, in addition to Ni, when the adaptive LASSO method was applied to identify $PM_{2.5}$ components associated with SBP. There is limited literature on the effects of ambient Na on cardiovascular health. Zanobetti et al. (2009) documented that Na^+ modified the relationship between $PM_{2.5}$ and emergency hospital admissions for cardiac diseases.

In the study, 7-day moving average $PM_{2.5}$ concentration ranged from 3.2 to 34.3 μg/m^3, while daily $PM_{2.5}$ was in the range of 1.2 ~ 44.8 μg/m^3 with a 99th percentile of 34 μg/m^3. Hence, we identified associations in a study population that was usually exposed to $PM_{2.5}$ concentrations below the current EPA daily ambient standard 35 μg/m^3 (EPA 2012). Associations with Ni were similar when we excluded observations with 7-day moving average PM concentration ≥ 12 μg/m^3. Our findings may suggest stricter air quality standards.

To date, many studies have investigated the biological mechanisms of the adverse effects of inhalation exposures to PM on cardiovascular diseases. Brook et al. (2010) summarized there are three potential pathways: 1) inducing pulmonary oxidative stress and inflammation via the release of proinflammatory mediators or vasculoactive molecules; 2) interacting with lung receptors or nerves to perturb systemic autonomic nervous system balance or heart rhythm; 3) PM or PM components being transmitting into the systemic circulation. Metals are typical PM components.

It has been documented that metals can enhance lung inflammation and injury (Ghio and Devlin 2001; Schaumann et al. 2004), which may be attributed to the metal-catalyzed oxygen stress via non-nitric oxide pathways (Dye et al. 1997). Nevertheless, mechanisms of cardiovascular effects of Ni have not been fully established. Previous studies have shown that metals in particles (e.g., Ni, V) could induce the activation of transcription factor NF-κB (a family of proteins that regulates DNA transcription in cellular responses such as immune, inflammatory response, and apoptosis), cell apoptosis and cell cycle regulation (Chen and Shi 2002; Goebeler et al. 1995; Quay et al. 1998). Although the clinical relevance is unclear, our finding that an IQR increase in Ni was associated with a 2.48/2.22 mm Hg increase in blood pressure may imply elevated risks of cardiovascular outcomes induced by Ni.

The major strengths in the study were highlighted as follows. 1) We used a novel approach, the adaptive LASSO, to investigate the relationship between $PM_{2.5}$ components and health outcomes. This method has advantages over conventional approaches. Typically, researchers examined effects of components by including all components in models or by using conventional selection procedures, such as stepwise selection. Linear regression with all components included may fail to detect any association as the collinearity among components reduce power, while conventional selection methods make no guarantee to select the right variables asymptotically. 2) To our knowledge, this is the first longitudinal cohort study that examined the effects of PM-related metals on blood pressure. The study population was geographically stable, well described and followed up since the enrollment in 1963. 3) We had daily concentrations of PM metals for more than 10 years. In previous studies, especially large national/multi-city studies, researchers usually used data from EPA Air Quality System that was sampling PM components every 3rd or 6th day (Dai et

al. 2014; Krall et al. 2013; Zanobetti et al. 2009), and hence had to face the challenge in lack of data.

On the other hand, there are several limitations in the study. Due to the use of stationary measures of $PM_{2.5}$ components, we were unable to capture the personal exposures of our subjects. Another limitation of our study is the potential measurement errors in blood pressure since blood pressure was measured only once at each study visit. Last, since the study population was limited to elderly men, most of whom were Caucasian, our findings cannot be directly generalized to women, younger men, or more diverse populations of elderly men. Subjects voluntarily continue to participate in the ongoing NAS study, so there may be volunteer bias if healthier people are more likely to participate. Also, there would be survivor bias if people who stay in the study are healthier than other people.

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Study 3. Differential DNA Methylation and PM2.5 Species in a 450K Epigenome-Wide Association Study

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Abstract

Background: Although there is growing evidence that exposure to ambient particulate matter is associated with global DNA methylation and gene-specific methylation, little is known regarding epigenome-wide change in DNA methylation in relation to particles, and less to their components.

Objectives: to examine epigenome-wide DNA methylation associated with $PM_{2.5}$ components in a longitudinal cohort.

Methods: Using the Illumina Infinium HumanMethylation450 BeadChip, we examined the relationship between one-year moving average $PM_{2.5}$ species (Al, Ca, Cu, Fe, K, Na, Ni, S, Si, V, and Zn) and DNA methylation at 484 613 CpG probes in a longitudinal cohort that included 646 subjects. Bonferroni correction was applied to adjust for multiple comparisons. Bioinformatic analysis of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment was also performed.

Results: We observed 20 Bonferroni significant (p-value $< 9.4 \times 10^{-9}$) CpGs for Fe, 8 for Ni, and 1 for V. Particularly, methylation at *SLFN11* (Schlafen Family Member 11) cg10911913 was positively associated with all the three species. The *SLFN11* gene codes for an interferon-induced protein that inhibits retroviruses and sensitizes cancer cells to DNA-damaging agents. Bioinformatic analysis suggests that gene targets may be relevant to pathways including cancers, signal transduction, cell growth and death. This is the first study on the epigenome-wide association of ambient particles species with DNA methylation.

Conclusions: We found that long-term exposure to particular components of ambient particle pollution, especially particles emitted during oil combustion, were associated with methylation changes in genes relevant to immune responses. Our findings provide insight into potential biological mechanisms on an epigenetic level.

Introduction

The associations between fine particulate matter ($PM_{2.5}$, particles with an aerodynamic size of ≤ 2.5 μm) and health outcomes are well established. It is estimated that $PM_{2.5}$ leads to 3.15 million premature deaths per year on a global scale (Lelieveld et al. 2015). Nationwide and multiple-community studies reported that exposure to $PM_{2.5}$ was linked with increased risk in hospital admissions and mortality in the U.S., and certain $PM_{2.5}$ species may be more toxic than others (Dai et al. 2014; Krall et al. 2013; Peng et al. 2009; Zanobetti et al. 2009).

DNA methylation is an epigenetic process in which a methyl group is added to deoxycytosine bases to form deoxymethylcytosine. Literature has shown that DNA methylation may be an important pathway linking particulate matter (PM) to health outcomes (Baccarelli and Bollati 2009; Belinsky et al. 2002; Chen et al. 2004; Chen et al. 2015; Madrigano et al. 2011). For example, changes in patterns of DNA methylation may be associated with processes leading to cardiovascular diseases (Baccarelli et al. 2010; Castro et al. 2003). There is growing evidence that exposure to PM is associated with global DNA methylation and gene-specific methylation (Bellavia et al. 2013; Chen et al. 2015; Madrigano et al. 2011; Madrigano et al. 2012); particularly, exposure to metal-rich PM in occupational settings (Fan et al. 2014; Kile et al. 2013). Yet, little is known regarding how DNA methylation changes on a genome-wide level.

Measurements of DNA methylation at cytosine-guanine dinucleotide (CpG) loci have recently become available; for instance, the Illumina Infinium HumanMethylation450 Beadchip (450K; Illumina Inc.) measures methylation at more than 450,000 CpG sites. This makes an epigenome-wide association study between particles and DNA methylation possible. Several studies have investigated the epigenome-wide association between smoking, an important PM source, and DNA

methylation. Using the 450K platform and Bonferroni correction, Joubert *et al*. (2012) identified 26 CpGs with methylation changes in newborns related to maternal smoking during pregnancy in a large Norwegian birth cohort. Specifically, the authors documented CpGs in *CYP1A1* (cytochrome p450 1A1) and *AHRR* (aryl-hydrocarbon receptor repressor), genes known to play a key role in the AhR (aryl hydrocarbon receptor) signaling pathway that mediates the detoxification of components of tobacco smoke. Zeilinger *et al*. (2013) conducted an epigenome-wide study comparing methylation levels among current, former, and never smokers in a population-based panel, and found widespread differences in the degree of site-specific methylation as a function of tobacco smoking. Moreover, they observed that the most significant associations with smoking were DNA methylation sites in *AHRR*.

To date, there are very few epigenome-wide association studies focusing on ambient particles. A recent study has identified several CpG sites associated with cumulative exposure to ambient particles in an epigenome-wide study among populations from Germany and the U.S. (Panni et al. 2016). Yet, no studies were reported regarding the toxicity of species. Such research would be of great importance because epigenome-wide association research based on DNA methylation microarrays could provide insight into epigenetic mechanisms of air particles and improve our understanding of human disease. In the current study, we, for the first time, examined the epigenome-wide association between $PM_{2.5}$ species and DNA methylation using data from a large longitudinal cohort.

Methods

Study population

Study subjects were participants of the Normative Aging Study (NAS) that was established in 1963 by the Department of Veterans Affairs. It is an ongoing longitudinal study of aging that enrolled 2280 community-dwelling, healthy men living in the Greater Boston area (Bell et al. 1972). Participants were free of known chronic medical conditions at enrollment and have undergone examinations every 3 to 5 years on a continuous rolling basis. All participants provided written informed consent. The study was reviewed and approved by the Institutional Review Boards of all participating institutions.

We restricted study subjects to those who provided DNA samples and who were Caucasian, who comprise the vast majority of our study population (approximately 97%). We then excluded observations that did not have complete information on exposure or covariates of interest. In the final analyses, samples from 646 subjects with 1031 total visits were included. Of the 646 subjects, 314 had one visit, 279 had two visits, and 53 had three visits. The flowchart of our study participants is shown in Figure 3-1.

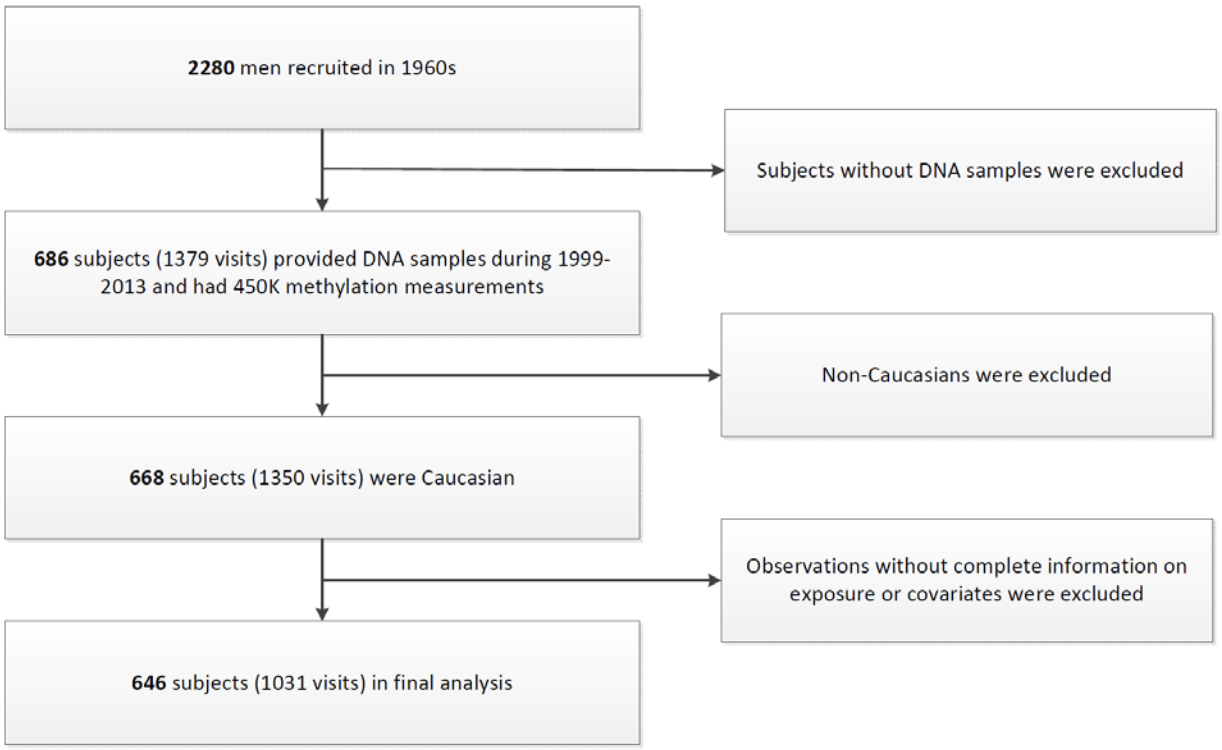

Figure 3-1. Flowchart of study participants.

DNA methylation

DNA samples were collected between 1999 and 2013. We used the QIAamp DNA Blood Kit (QIAGEN, CA, USA) to extract DNA from buffy coat, and performed bisulfite conversion with the EZ-96 DNA Methylation Kit (Zymo Research, CA, USA). To minimize batch effects, we randomized chips across plates and randomized samples based on a two-stage age stratified algorithm so that age distributed similarly across chips and plates.

We measured DNA methylation of CpG probes using Illumina's Infinium HumanMethylation450 BeadChip (Bibikova et al. 2011). Quality control analysis was performed, where samples with >

1% of probes have a detection *p*-value of > 0.05 were removed, and probes with > 5% samples have beadcount < 3 or with > 1% samples have a detection *p*-value of > 0.05 were removed. The remaining samples were preprocessed using the Illumina-type background correction (Triche et al. 2013) and normalized with the dye-bias (Davis et al. 2015) and BMIQ3 adjustments (Teschendorff et al. 2012), which were used to generate methylation values. Extreme outliers, defined by Tukey's method (i.e., < 25^{th} percentile – 3 × IQR or > 75^{th} percentile + 3 × IQR), were trimmed. As a result, 484 613 CpG probes were in the working set.

We used the β value to indicate methylation level at each CpG site. β value represents the ratio of the fluorescence of the methylated signal to the combined methylated and unmethylated signals, that is, β = intensity of the methylated signal (M) / (intensity of the unmethylated signal (U) + intensity of the methylated signal (M) + 100) (Bibikova et al. 2011).

Air pollution

We measured daily concentrations of $PM_{2.5}$ mass and species at the stationary monitoring supersite at the Harvard University Countway Library, Boston, MA, USA. $PM_{2.5}$ was assessed by the Tapered Element Oscillating Microbalance (TEOM 1400a, Rupprecht & Patashnick Co.) and species by the Energy Dispersive X-ray Fluorescence Spectrometer (Epsilon 5, PANalytical).

To estimate long-term effects of air pollution on DNA methylation, we calculated one-year moving average concentrations for $PM_{2.5}$ and the following eleven species: Al, Ca, Cu, Fe, K, Na, Ni, S, Si, V, and Zn. These species are representative of different sources of particles: Al, Ca, Fe, and Si are major components of road dust; Cu and Zn originate from traffic emissions; Ni and V are tracers of oil combustion; S is a regional pollutant that is associated with coal combustion and to

a lesser extent with metallurgic activities; Na is a major component of sea salt particles; K originates from wood burning and soil.

Statistical analysis

For each specific CpG site, we applied a linear mixed-effects model as described below,

$$E(Y_{ij}) = \beta_0 + \beta_1 * PM_{2.5ij} + \beta_2 * Comp_{ij} + \beta_3 * Age_{ij} + \ldots + \mu_i, \quad [3.1]$$

where, Y_{ij} is the methylation level of subject i at visit j, $PM_{2.5ij}$ is one-year moving average $PM_{2.5}$ concentration previous to visit j, $Comp_{ij}$ is the one-year moving average component, Age_{ij} is subject i's age at visit j (same for other covariates, we used value at each visit in analyses), and μ_i is the random intercept that accounts for correlation within subject.

In the analysis, we controlled for the following covariates *a priori* based on literature (Madrigano et al. 2011; Mostofsky et al. 2012; Zeilinger et al. 2013; Zhu et al. 2012): total $PM_{2.5}$ mass, cell proportions ($CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, natural killer cells, B cells, monocytes) estimated by the Houseman *et al*. method (Aryee et al. 2014; Houseman et al. 2012; Reinius et al. 2012), age, body mass index (BMI, computed as weight [in kilograms] divided by height [in square meters]), cigarette pack years, smoking status (never, ever), alcohol consumption ($\geq$ 2 drinks/day, < 2 drinks/day), years of education, year and season (spring: March-May, summer: June-August, fall: September-November, winter: December-February). In adjusting for cell proportions, we did not include granulocytes, i.e., the cell type with the largest proportion, to avoid the identifiability problem. Potential batch effects were also considered, including plate, position of chip on plate, row and column position on chip.

Since overall type I error inflates as the number of statistical tests increases, known as multiple comparisons, we used Bonferroni correction, which has been widely used in similar studies (Huang da et al. 2009a, b; Li et al. 2012), accounting for number of CpG sites and exposures tested. As a result, the adjusted level of significance was 9.4×10^{-9}, i.e., 0.05 / (484 613 × 11) in the current study. After identifying significant CpGs, we further checked whether they were non-unimodal in the dip test (Hartigan and Hartigan 1985), were cross-reactive (Chen et al. 2013), or contained SNPs in the last 10 bases from those of European populations (Abecasis et al. 2012).

All statistical analyses were performed using R 3.1.2 (R Core Team 2014).

Bioinformatic analysis

We performed KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis (Kanehisa et al. 2002) using the genes targets of CpGs, identified by the Illumina annotation assignment, with Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg 1995) < 1% and those with FDR < 5%, respectively. Bioinformatic analysis was carried out using DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources 6.7 (Huang da et al. 2009a, b). Briefly, DAVID consists of an integrated biological knowledgebase with wide annotation content coverage. It allows us to find out the most relevant pathways associated with the gene lists we provided.

Results

Characteristics of study subjects are presented in Table 3-1. At baseline, the mean age was 74 (SD = 7) years. The study population was well-educated, with a mean of 15 (SD = 3) years of education. Furthermore, 186 of the 646 (28.8%) individuals never smoked, and 131 (20.3%) had two or more drinks per day.

Table 3-1. Characteristics of the study population.

Variable	**First visit (n = 646)**	**All visits (n = 1031)**
Mean ± SD		
Age (years)	73.7 ± 6.8	74.7 ± 6.8
BMI (kg/m^2)	28.0 ± 4.1	28.0 ± 4.2
Education (years)	15.1 ± 3.0	15.2 ± 3.0
Cigarette smoking (pack years)	21.3 ± 25.5	21.3 ± 25.2
Number (%)		
Smoking status		
Never	186 (28.8)	298 (28.9)
Ever	460 (71.2)	733 (71.1)
Alcohol consumption (drinks/day)		
≥ 2	131 (20.3)	200 (19.4)
< 2	515 (79.7)	831 (80.6)

Table 3-2 shows the descriptive summary of concentrations of $PM_{2.5}$ mass and species. During the study period 1999 – 2013, the annual average $PM_{2.5}$ concentration was 10.5 (SD = 1.1) $\mu g/m^3$. Among the examined species, S accounted for the largest mass proportion (10%). The oil combustion tracers, Ni and V, accounted for 0.03% and 0.04% of $PM_{2.5}$ mass. Their mean concentrations were 0.0037 (SD = 0.0013) $\mu g/m^3$ and 0.0041 (SD = 0.0012) $\mu g/m^3$, respectively.

Table 3-2. Summary of one-year moving average $PM_{2.5}$ mass and species across all study visits (year 1999 - 2013).

Pollutant	**Mean ± SD (μg/m^3)**	**IQR (μg/m^3)**	**Proportion of the $PM_{2.5}$ mass (%)**
$PM_{2.5}$ mass	10.5 ± 1.1	1.9	-
Coal combustion			
S	1.11 ± 0.13	0.08	10
Sea salt			
Na	0.20 ± 0.01	0.01	1.8
Road dust			
Al	0.051 ± 0.008	0.009	0.5
Ca	0.032 ± 0.005	0.007	0.3
Fe	0.069 ± 0.010	0.012	0.6
Si	0.075 ± 0.016	0.022	0.7
Wood burning			
K	0.041 ± 0.002	0.003	0.4
Traffic			
Zn	0.013 ± 0.003	0.004	0.1
Cu	0.0035 ± 0.0003	0.0004	0.03
Oil combustion			
Ni	0.0037 ± 0.0013	0.0011	0.03
V	0.0041 ± 0.0012	0.0015	0.04

Table 3-3 presents CpGs that reached Bonferroni-corrected significance (p-value < 9.4×10^{-9}) in relation to $PM_{2.5}$ species. After excluding CpGs that were non-unimodal in the dip test, were cross-reactive, or contained SNPs in the last 10 bases, we observed 20 significant CpGs for Fe, 8 for Ni, and 1 for V. Particularly, *SLFN11* cg10911913 was significant for all the three metals. The ratio of methylation on this CpG site is expected to increase by 0.073, 0.031, and 0.044 per IQR increase in one-year moving average Fe, Ni, and V, respectively. We also found that *CASZ1* cg16238819, *CUEDC2* cg06024834, *ONECUT1* cg15446043, and *FOXO4* cg12453500 were significant for both Fe and Ni. To illustrate the epigenome-wide association between $PM_{2.5}$ species and

methylation, we plotted the -$\log_{10}$(*p*-value) from the mixed-effects models for all the 484,613 CpG sites in Figure 3-2. The horizontal line represents the significance threshold with Bonferroni correction (p-value < 9.4×10^{-9}). We originally found 22 significant CpGs for Fe, 9 for Ni, and 1 for V, as shown in Figure 3-1. After excluding CpGs that were non-unimodal in the dip test, were cross-reactive, or contained SNPs in the last 10 bases, 20 CpGs remained significantly associated with Fe, 8 with Ni, and 1 with V.

Table 3-3. Genome-wide association of one-year moving average $PM_{2.5}$ species with DNA methylation: CpGs with Bonferroni-corrected statistical significance (p-value < 9.4 × 10-9).

Species	**Chr**	**Gene**	**CpG**	**Est**	**Coef**	**SE**	***p*-value**
Fe	1	*DHRS3*	ch.1.471396R	0.053	0.089	0.015	2.37E-09
	1	*CASZ1*	cg16238819	0.076	0.126	0.021	6.94E-09
	6	*SLC39A7;RXRB*	cg24319547	0.031	0.052	0.009	2.20E-09
	6	*KIAA1949*	cg08052856	0.072	0.120	0.020	2.58E-09
	6	*CDC5L*	cg22877480	0.068	0.113	0.019	8.30E-09
	8	*FBXO25*	ch.8.7249R	0.062	0.103	0.017	4.40E-09
	10	*CUEDC2*	cg06024834	0.039	0.065	0.011	1.98E-09
	11	*RPS6KB2*	cg26457823	0.035	0.059	0.010	3.82E-09
	11	*RPS25;TRAPPC4*	cg05223675	0.077	0.128	0.021	6.97E-09
	14	*SMEK1*	cg01561368	0.024	0.040	0.007	6.20E-09
	15	*ONECUT1*	cg15446043	0.146	0.243	0.039	1.37E-09
	15	*TPM1*	cg12302829	0.039	0.065	0.011	2.72E-09
	17	*SLFN11*	cg10911913	0.073	0.122	0.018	1.84E-10
	17	*CYTH1*	cg26525457	-0.076	-0.126	0.020	1.26E-09
	18	*RNF138*	cg16078886	0.065	0.108	0.018	2.55E-09
	19	*MYPOP*	cg03766703	-0.119	-0.198	0.033	6.91E-09
	19	*REEP6;PCSK4*	cg13970591	0.222	0.370	0.063	8.10E-09
	21	*C21orf57*	cg10434947	0.043	0.072	0.012	4.32E-09
	22	*CBY1;LOC646851*	cg11003278	0.050	0.084	0.013	4.71E-10
	X	*FOXO4*	cg12453500	0.059	0.098	0.014	2.89E-11
Ni	1	*CASZ1*	cg16238819	0.032	1.062	0.179	8.25E-09
	10	*CUEDC2*	cg06024834	0.017	0.560	0.089	1.10E-09
	15	*ONECUT1*	cg15446043	0.059	1.950	0.330	8.31E-09
	16	*WDR90*	cg02734472	0.035	1.168	0.194	4.61E-09
	17	*SLFN11*	cg10911913	0.031	1.046	0.156	7.56E-11
	18	*RNF138*	cg16078886	0.029	0.973	0.148	1.91E-10
	19	*SEC1;DBP*	cg13402773	0.056	1.850	0.310	6.22E-09
	X	*FOXO4*	cg12453500	0.022	0.739	0.120	2.10E-09
V	17	*SLFN11*	cg10911913	0.044	1.103	0.174	6.81E-10

Abbreviation: Chr: Chromosome; Est: Estimate of change in β value per IQR increase in the species; Coef: Regression coefficient in the mixed-effects models, with adjustment for $PM_{2.5}$ mass, Houseman estimated cell proportions ($CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, natural killer

cells, B cells, monocytes), age, BMI, cigarette pack years, smoking status, alcohol consumption, education, year, season, and batch effects (plate, position of chip on plate, row and column position on chip); SE: Standard error for regression coefficient.

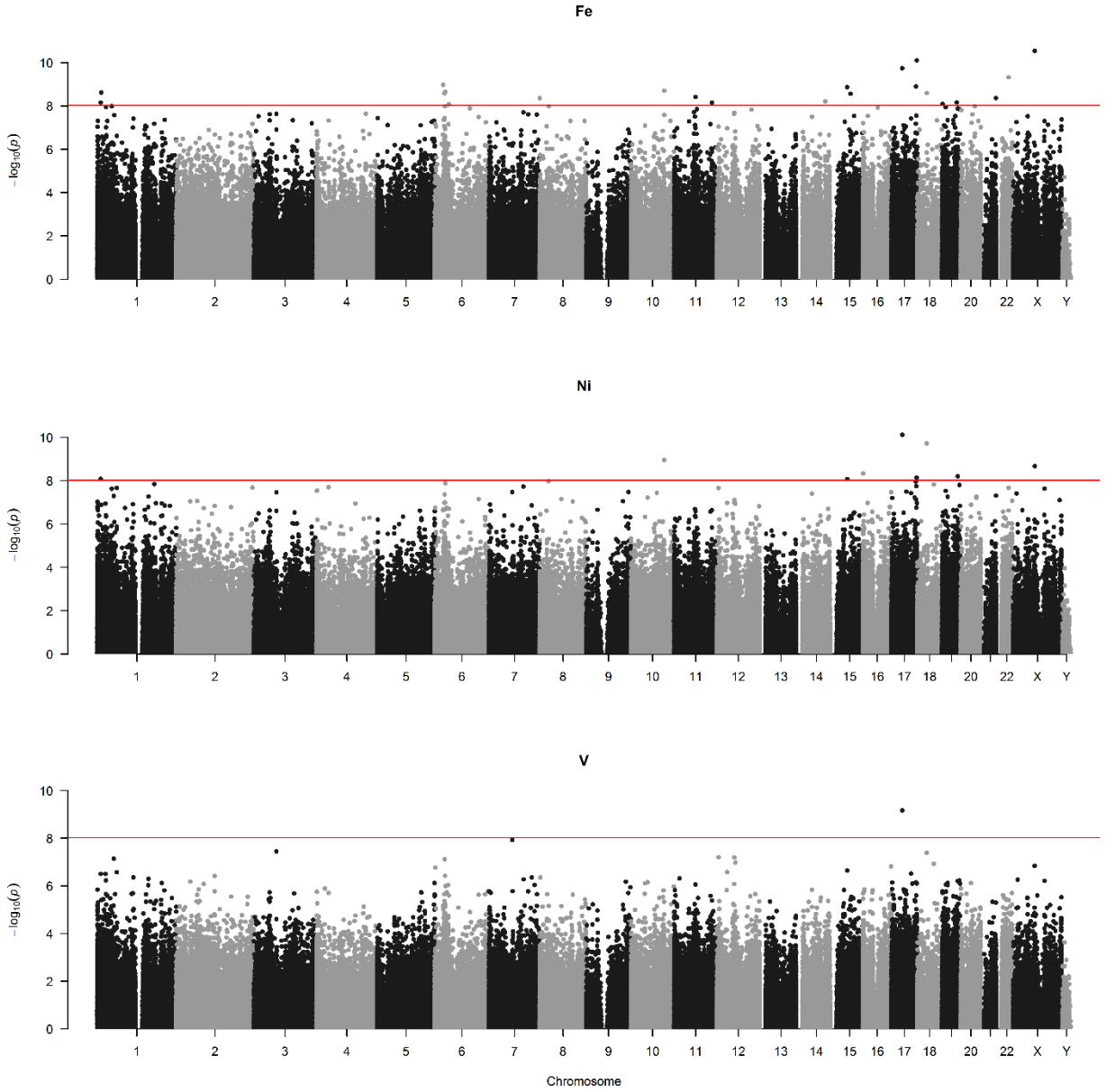

Figure 3-2. Figure 3 2. Genome-wide association of one-year moving average $PM_{2.5}$ species with DNA methylation, displayed by Manhattan plots.

Results of the bioinformatic analysis are shown in Figure 3-3. We conducted KEGG pathway enrichment analysis for gene targets of CpGs with FDR < 1% and < 5%, respectively. Pathways

whose enrichment FDRs were less than 5% were considered significant and indicated by colored chunks in the figure. In analysis for gene targets of CpGs with FDR < 1%, we identified nine significant KEGG pathways. Three of them (i.e., “pathways in cancer”, “non-small cell lung cancer”, and “glioma”) belong to the “Cancers” network. Results with gene targets of CpGs with FDR < 5% suggest that those genes may also be linked to other cancers (e.g., myeloid leukemia) and pathways in the immune system. Of interest among the other pathways identified are the diabetes pathway and MAPK (mitogen-activated protein kinases) signaling pathway which is involved in cellular functions including cell proliferation, differentiation and migration.

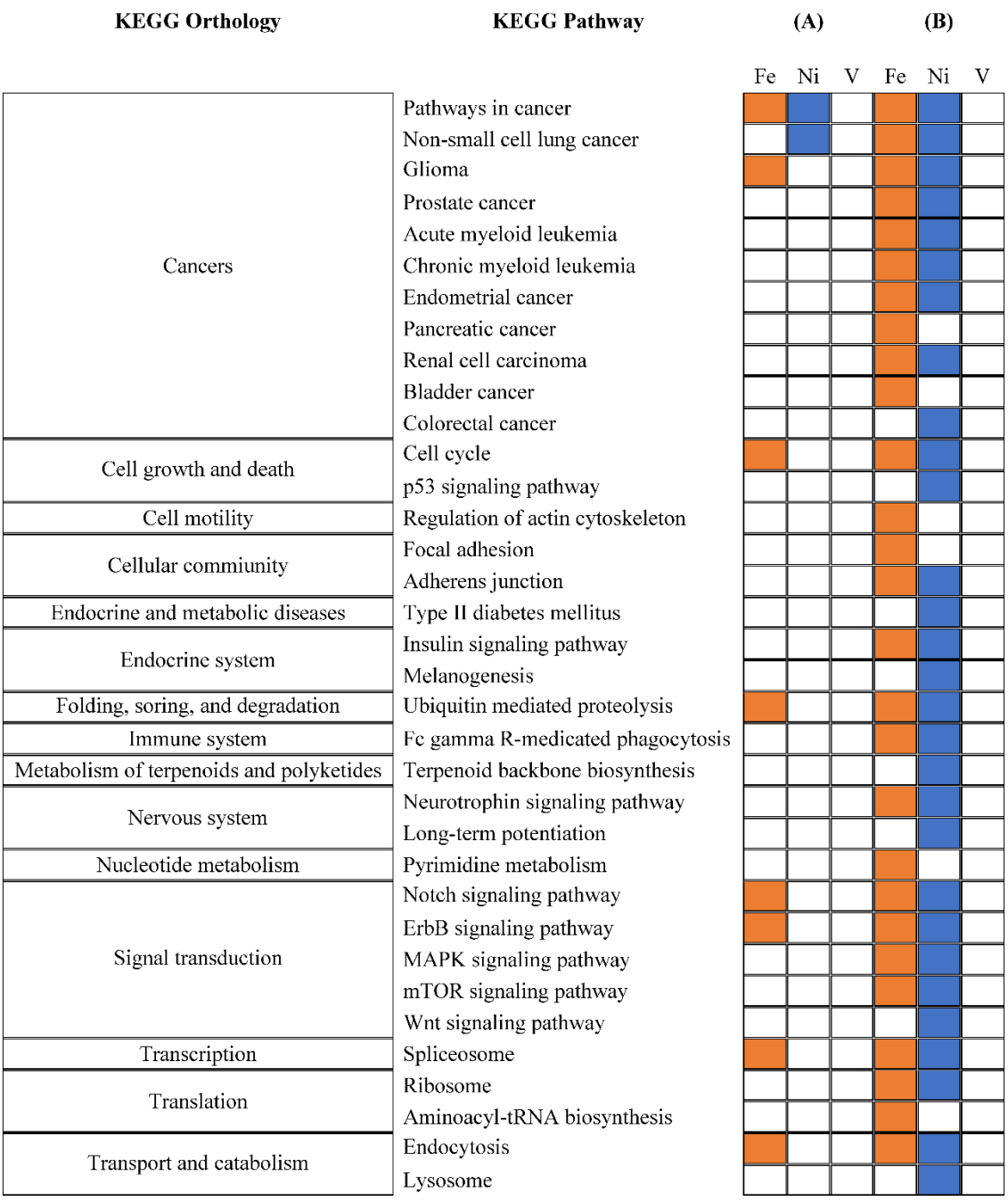

Figure 3-3. KEGG pathway enrichment analysis for (A) gene targets of CpGs with FDR < 1%, and (B) gene targets of CpGs with FDR < 5%.

Discussion

In this study, we investigated the relationship between $PM_{2.5}$ species and epigenome-wide DNA methylation in a large longitudinal cohort of elderly men. To the best of our knowledge, this is the first study that examined epigenome-wide association between ambient $PM_{2.5}$ components and DNA methylation. We adjusted for the multiple tests from 11 components and the 484 613 CpGs analyzed. We observed that 20 CpGs met the conservative Bonferroni-corrected level of significance (p-value $< 9.4 \times 10^{-9}$) in the analysis for Fe, 8 for Ni, and 1 for V. In addition, we found that all three metals were associated with cg10911913 on the *SLFN11* gene. Our bioinformatic analysis suggests that gene targets of CpGs with FDR < 1% may be relevant to pathways including cancers, signal transduction, cell growth and death.

To date, only a limited number of research studies have examined the association of ambient particles with DNA methylation. Most of them used surrogates, such as repetitive elements, for global DNA methylation or focused on methylation in candidate genes. The main shortcomings of these studies are: 1) methylation levels measured by surrogates do not consider the location in the genome and the patterns of methylation at surrogates may not well represent the patterns of gene methylation, and 2) use of candidate genes can only reveal methylation changes in specific genes selected *a priori* and miss the genes that exhibit the most profound effects.

One previous study regarding PM exposure and DNA methylation reported that exposures to 90-day moving average black carbon and sulfate particles concentrations were associated with hypomethylation of the long interspersed nucleotide element-1 (LINE-1) and the short interspersed nucleotide element *Alu* (Madrigano et al. 2011). Baccarelli *et al*. (2009) found decreased LINE-1 methylation in association with 7-day moving average black carbon and $PM_{2.5}$ levels, whereas no

association was observed for *Alu* methylation. A controlled human exposure experiment has observed hypomethylation of *Alu* and *TLR4* (toll-like receptor 4) gene among 15 healthy adults exposed to fine concentrated ambient particles (CAPs) for 130 minutes (Bellavia et al. 2013). Recently, a longitudinal study reported a relationship between decreased methylation at three CpG loci located in the *NOS2A* (nitric oxide synthase 2, inducible) gene and short-term exposures to organic carbon, elemental carbon, NO_3^- and NH_4^+ among 28 patients with chronic obstructive pulmonary disease (Chen et al. 2015). A more recent epigenome-wide study found variations in DNA methylation associated with short- and mid-term $PM_{2.5}$ exposure among Germany and U.S. cohorts (Panni et al. 2016).

The most consistent finding of our study was the significant increases in methylation at *SLFN11* cg10911913 that was associated with one-year moving average concentrations of $PM_{2.5}$ species Fe, Ni, and V. *SLFN* (Schlafen Family Member) genes encode a family of proteins that have been implicated in the regulation of cell growth and T cell development (Geserick et al. 2004; Schwarz et al. 1998). *SLFN11*, a member of the *SLFN* family, potently and specifically abrogates the production of retroviruses, e.g., human immunodeficiency virus (HIV-1) (Li et al. 2012). Using cells with endogenously high and low *SLFN11* expression and siRNA-mediated silencing, Zoppoli *et al*. (2012) found that *SLFN11* causally determines cell death and cell cycle arrest in response to DNA-damaging agents in cancer cells from different tissues of origin. *SLFN11* cg10911913 is located in a region whose function is promoter associated. Our results showed that the methylation in this CpG site was positively associated with one-year moving average $PM_{2.5}$ species Fe, Ni, and V. Our analysis suggests that exposure to ambient particles, especially those from oil combustion, was associated with hypermethylation of CpGs on *SLFN11*, which might lead to its downregulation and thus declines in inhibiting retroviruses and increased DNA damage to cancer

cells. This hypothesis is consistent with recent literature on air-pollution-induced activation of viral sequences integrated in the human genome. A study of truck drivers and office workers in Beijing, China showed a significant association of exposure to elemental carbon – taken as a marker of traffic particle exposure – with altered expression of viral microRNAs, possibly reflecting reactivation of latent retroviral sequences integrated in the host DNA (Hou et al. 2015). In addition, the International Agency for Research on Cancer has recently classified particulate air pollution as a known human carcinogen (Loomis et al. 2013), and this result is consistent with a carcinogenic potential.

We also observed that methylation at *ONECUT1* cg15446043 was positively associated with Fe and Ni. *ONECUT1* (one cut homeobox 1) can inhibit hepatitis B virus, a small enveloped DNA virus, gene expression and DNA replication via both transcriptional and post-transcriptional mechanisms (Hao et al. 2015). Hypermethylation at this CpG associated with PM exposures may depress *ONECUT1* expression, and thus lead to an increased risk of viral infections.

In addition, we found that long-term exposure to Fe was linked to methylation in other genes functioning on immune cells, including *CYTH1* and *RPS6KB2*. *CYTH1* (cytohesin 1) regulates adhesion and transendothelial migration of monocytes, T lymphocytes, and dentritic cells; it is also a key regulator of neutrophil adhesion to endothelial cells and to components of extracellular matrix (El azreq and Bourgoin 2011; Kolanus 2007). *RPS6KB2* (ribosomal protein S6 kinase) plays an important role in neutrophilic differentiation and neutrophilic proliferation in HL-60 (Human promyelocytic leukemia) cells. Hence, methylation changes at pertinent CpG sites of these two genes may affect immune functions.

Interestingly, Fe in our data was associated with methylation levels of CpGs in genes that regulate cell cycle, such as *CDC5L* cg22877480 and *RPS6KB2* cg26457823 (Boyer et al. 2008; Graub et al. 2008). *CDC5L* (cell division cycle 5-like) is a crucial regulator of cell cycle G2/M progression and a component of pre-mRNA processing, hence previous research has suggested it as a target for cancer therapy (Graub et al. 2008; Mu et al. 2014); *RPS6KB2* (ribosomal protein S6 kinase) is also highly active in the G2 and M phases (Boyer et al. 2008). Changes in methylation in these genes might lead to unguarded cell proliferation. This finding is consistent with the results of our bioinformatic analysis, in which we found that the gene targets may be related to biological pathways including cancer, cell growth and death.

The DAVID bioinformatic analysis also indicated that Fe and Ni were associated with type II diabetes mellitus, insulin signaling pathways, and the MAPK pathway. This is consistent with previous work. For instance, long-term $PM_{2.5}$ exposure resulted in systemic inflammation, increased visceral adiposity and insulin resistance in mice on a high fat diet (Sun et al. 2009). In mice on normal diets, PM exposure was a risk factor for the development of type II diabetes as it induced insulin resistance and impaired glucose tolerance (Xu et al. 2011). Similarly in humans, long-term exposure to particulate matter was linked to increased risk of type II diabetes in a large cohort study (Weinmayr et al. 2015). We have also reported in the NAS cohort that particulate air pollution was associated with changes in methylation in the MAPK pathway, but were unable to analyze metal components as in this study (Carmona et al. 2014).

Earlier, we found particles from oil combustion were associated with markers of inflammation and endothelial dysfunction, and with increased blood pressure in the NAS population (Dai et al. 2015; Dai et al. 2016). The current study may provide potential mechanisms on an epigenetic level: a proinflammatory stimulus such as air pollution modifies methylation in genes relevant to immune

responses, inducing inflammation and endothelial dysfunction, thereby influencing cardiovascular health.

Our study has several strengths. First, this is the first study that examined the effects of ambient particulate matter on epigenome-wide DNA methylation. Previous studies often estimated DNA methylation associated with ambient PM using surrogates for global methylation or gene-specific methylation at candidate genes. An epigenome-wide study like ours offers a novel perspective on such an association, and implicates potential biological mechanisms on an epigenetic level. Second, we used a stringent Bonferroni correction to adjust the level of significance, which accounted both for the 11 $PM_{2.5}$ species and for the number of CpG sites analyzed. Although we observed a small number of significant CpGs due to the strict correction, the probability of false positive is expected to remain appropriate. Third, the study population was geographically stable and well followed since the enrollment in 1963.

On the other hand, this study has several notable limitations. We do not have information on gene expression, and thus we cannot confirm the expected functional implications of the methylation changes. Further research on gene expression in relation to PM exposures is needed. Additionally, we are unable to rule out residual confounding. The bioinformatic analysis of pathways used annotations of methylation sites within genes and thus genes that include more sites on the 450K array, such as those involved in cancer, are more likely to arise in pathway results (Harper et al. 2013). Last but not least, there exists the generalizability issue because the study population consisted of elderly Caucasian men. One should consider factors like age, sex, and race when generalizing our findings to other populations.

In summary, we examine the association of long-term exposure to $PM_{2.5}$ species with differential DNA methylation in a 450K epigenome-wide association study using a large longitudinal cohort. Our study confirmed previous research on the association of ambient $PM_{2.5}$ mass on epigenome-wide DNA methylation (Panni et al. 2016). It also provides a possible link between the effects and species; particularly, it suggests that particles from oil combustion were associated with methylation changes in genes relevant to immune responses.

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Conclusions

The findings showed an increased risk of mortality and blood pressure associated with $PM_{2.5}$, which varied with species. In particular, silicon and calcium from road dust as well as sulfur from coal burning were associated with higher risk of mortality, while nickel from oil combustion were responsible for the effects of $PM_{2.5}$ mass on elevated blood pressure. Epigenome-wide association study observed differential DNA methylation: long-term exposure to particular components of ambient particle pollution, especially particles emitted during oil combustion, were associated with methylation changes in genes relevant to immune responses.

In conclusion, mass alone might not be sufficient to evaluate the health effects of particles. Further research is needed to confirm the findings, assess generalizability to other populations, and identify potential mechanisms for $PM_{2.5}$ species.