RESEARCH PAPER

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Air pollution and gene-specific methylation in the Normative Aging Study

Association, effect modification, and mediation analysis

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Abbreviations: CRP, C-reactive protein; CpG, Cytosine-phosphodiester-guanine; DAG, Directed acyclic graph; GEE, Generalized estimating equations; ICAM-1, Intercellular adhesion molecule 1; IFN-γ, Interferon gamma ; IL-6, Interleukin 6 ; IPCW, Inverse probability censoring weights; LINE-1, Long Interspersed Nucleotide Element 1; %5mC, Percent of 5-methylated cytosine; F3, Tissue factor; TLR-2, Toll-like receptor 2; VCAM-1, Vascular cell adhesion protein 1

The mechanisms by which air pollution has multiple systemic effects in humans are not fully elucidated, but appear to include inflammation and thrombosis. This study examines whether concentrations of ozone and components of fine particle mass are associated with changes in methylation on tissue factor (*F3*), interferon gamma (*IFN-γ*), interleukin 6 (*IL-6*), toll-like receptor 2 (*TLR-2*), and intercellular adhesion molecule 1 (*ICAM-1*). We investigated associations between air pollution exposure and gene-specific methylation in 777 elderly men participating in the Normative Aging Study (1999–2009). We repeatedly measured methylation at multiple CpG sites within each gene's promoter region and calculated the mean of the position-specific measurements. We examined intermediate-term associations between primary and secondary air pollutants and mean methylation and methylation at each position with distributed-lag models. Increase in air pollutants concentrations was significantly associated with *F3*, *ICAM-1*, and *TLR-2* hypomethylation, and *IFN-γ* and *IL-6* hypermethylation. An interquartile range increase in black carbon concentration averaged over the four weeks prior to assessment was associated with a 12% reduction in *F3* methylation (95% CI: -17% to -6%). For some genes, the change in methylation was observed only at specific locations within the promoter region. DNA methylation may reflect biological impact of air pollution. We found some significant mediated effects of black carbon on fibrinogen through a decrease in *F3* methylation, and of sulfate and ozone on ICAM-1 protein through a decrease in *ICAM-1* methylation.

Introduction

Although levels of most air pollutants have decreased over the last decade, air pollution remains an important public health issue. Moreover, the US population is aging and the elderly constitutes a population susceptible to air pollution exposure. Particulate pollution is known to increase cardiovascular morbidity and mortality[1] and the relative contribution of particle components is still unclear. Animal and human studies have linked high air pollution levels to thrombosis and systemic inflammation.[2-5]

Recent research has identified a new biological mechanism to explain adverse health effects from air pollution: epigenetics.[6,7] Epigenetics refers to chromosome changes that do not modify the genetic code, but influence its expression. The most frequently examined epigenetic mechanism is called DNA methylation because it involves methylation of cytosine in CpG pairs. A recent study suggested that DNA methylation is a mechanism that cells use to control gene expression in a switch-like manner.[8] DNA methylation has been associated with health outcomes[9-12] that have, in turn, been related to particles and ozone exposure.[1] This raises the question of whether DNA methylation plays a role in the air pollution adverse effects on cardiovascular diseases.

Changes in methylation have been associated with exposure to lead[13,14] and air pollution.[15-19] Short- and intermediate-term exposures to black carbon and particles over one week have been associated with inducible nitric oxide synthase hypomethylation.[16,20-22] In addition, black carbon exposure over weeks or months has been related to Long Interspersed Nucleotide Element 1 (*LINE-1*) hypomethylation.[17,23] However, the literature on the effects of air pollution on methylation remains limited.

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We investigated whether air pollution exposures are related to methylation on genes related to two cardiovascular pathways: coagulation and inflammation in the elderly. We focused on five genes: tissue factor (*F3*), interferon gamma (*IFN-γ*), interleukin 6 (*IL-6*), toll-like receptor 2 (*TLR-2*), and intercellular adhesion molecule 1 (*ICAM-1*). We examined whether the association between air pollution and gene-specific methylation varied by participants' characteristics. We also investigated whether the association between air pollution and cardiovascular-related biomarkers was mediated via a change in gene-specific methylation.

Results

Descriptive statistics

At baseline, the median age of the study population was 72 years (**Table 1**). Also 27% of the participants were obese, 14% were diabetics, and only 4% were current smokers. Participants' characteristics varied according to their total number of visits: individuals with more visits seemed "healthier" than participants with fewer visits.

Boston has a continental climate with direct influences from the ocean. While it is mostly cold and dry in winter, it is usually warm and humid in summer. Ambient air pollutants levels in Boston are mostly below EPA standards. Over the 1999–2009 study period, the 24-h $PM_{2.5}$ mass concentrations exceeded the daily standard of 35 $\mu g/m^3$ only during 13 days in summer 2002. Summary statistics of the weather and air pollution distributions during the study period are presented in **Table S1**. Ozone was negatively correlated with particle number (Correlation = -0.39) and black carbon (Correlation = -0.14) (**Table S2**). We observed a decrease over time of the correlations between the weekly averaged air pollutant concentrations (**Table S3**).

The distributions of the gene-specific methylation varied according to genes: *IFN-γ* methylation distribution was wider compared with that of *F3* methylation (**Table S4**). The gene-specific distributions were concentrated around low or high values, except for *IL-6* methylation.

Main associations

Air pollutants levels were associated with significant changes in gene-specific methylation (**Fig. 1A and B**). We observed significant negative associations between markers of traffic such as particle number (1st week) and black carbon (3rd and 4th weeks) and *F3* methylation. For instance, an interquartile range (IQR) increase in particle number (which corresponds approximately to an increase of 15 000 particles per cm^3) over the 1st week preceding each participant's methylation measurement was associated with an 18% reduction in *F3* methylation (95% confidence interval [CI]: –29% to –4%). In contrast, the secondary pollutants sulfate and ozone were not associated with *F3* methylation. Ozone exposures over the 2nd to 4th weeks were negatively related to *ICAM-1* methylation. In addition, black carbon and ozone exhibited opposite associations with *ICAM-1*, *IFN-γ*, and *IL-6* methylation.

Our main analysis indicated temporal variation across lags in the association between air pollution exposure and DNA methylation. For instance, we observed an earlier decrease in *F3* methylation for particle number (1st week) compared with black carbon (3rd and 4th weeks), as well as a negative association between ozone and *ICAM-1* methylation after the 2nd week of exposure.

Air pollutants associations over the 4-week period preceding each medical visit are presented in **Table 2**. For example, an interquartile range increase in black carbon concentration was associated with a 12% reduction in *F3* methylation (95% CI: –17% to –6%).

We also examined whether some participants' characteristics were related to gene-specific methylation (**Table S5**). Compare with individuals who never smoked, former smokers had higher *IL-6* methylation and lower *TLR-2* and *IFN-γ* methylation. Age was also positively associated with *TLR-2* methylation.

Analyses of effect modification and mediation

We did not observe any effect modification by smoking status, except for the association between particle number and *IL-6* methylation which was stronger in participants who never smoked (methylation mean ratio for an IQR increase in particle number = 2.877 [95% CI: 1.221, 4.534]), compare with former and current smokers (methylation mean ratio = 0.886 [95% CI: -0.770, 2.543]) (**Table 3**). The association between air pollution and gene-specific methylation did not vary by obesity status (**Table S6**) or age category (**Table S7**). The air pollution association with gene-specific methylation was fairly similar according to baseline participants' levels of *LINE-1* and *Alu* methylation, except for ozone, for which results were not consistent (**Table S8**).

We conducted mediation analyses and found some significant mediated effects of black carbon on fibrinogen through a decrease in *F3* methylation (estimate: 0.012, 95% CI: [0.000, 0.024]), and of sulfate and ozone on ICAM-1 protein through a decrease in *ICAM-1* methylation (estimates: 0.007, 95% CI: [0.000, 0.014] and 0.037, 95% CI: [0.003, 0.072], respectively) (**Table 4**).

Sensitivity analyses

We found similar results when we assumed a distributed-lag model with constant lag associations within weeks for air pollution, temperature, and relative humidity (**Fig. 1A and B**) vs. a distributed-lag model constrained by natural splines with 3 degrees of freedom.

The magnitude and significance of the air pollution associations with gene-specific methylation differed by CpG positions where the methylation was measured (**Table S9**). For example, the signals of particle number, black carbon, and sulfate were the strongest at position 4 within the promoter region of *F3*, whereas the signal was the greatest at position 2 for ozone.

In addition, we examined the robustness of our main findings considering additional statistical approaches (**Table S10**). After fitting co-pollutant models, negative associations between traffic-related pollutants and *F3* methylation remained significant after adjusting for ozone. The ozone association estimate with *F3* methylation became significant when we controlled for particle number. Similarly, the co-pollutant models suggested that changes in *ICAM-1* methylation were related to particle number but not to ozone. Moreover, our results were not affected by residual confounding of smoking intensity captured by cigarette

Table 1. Demographical characteristics of the NAS participants across visits

	Age (years) [5th, 50th, 95th percentiles]	% of neutrophils [5th, 50th, 95th percentiles]	% of lymphocytes [5th, 50th, 95th percentiles]	Obesity	Statin user	Diabetics	Smoking [Never, Former, Current]
Baseline (n = 777)	[62, 72, 84]	[48, 62, 74]	[15, 26, 38]	27%	36%	14%	[29%, 67%, 4%]
$N_{missing}$	0	22	22	0	0	0	0
Among participants having one visit (n_1 = 221)							
Visit 1	[64, 76, 88]	[48, 63, 77]	[13, 25, 37]	30%	40%	18%	[26%, 70%, 4%]
Among participants having two visits (n_1 = 217)							
Visit 1	[60, 73, 83]	[47, 62, 74]	[15, 25, 40]	28%	35%	16%	[26%, 69%, 5%]
Visit 2	[66, 77, 86]	[48, 64, 75]	[14, 24, 37]	27%	54%	19%	[26%, 70%, 4%]
Among participants having three visits (n_3 = 216)							
Visit 1	[62, 71, 82]	[47, 62, 72]	[16, 26, 39]	25%	36%	9%	[29%, 68%, 3%]
Visit 2	[66, 74, 86]	[48, 62, 74]	[15, 26, 38]	26%	52%	13%	[28%, 69%, 3%]
Visit 3	[69, 78, 89]	[48, 62, 76]	[13, 25, 39]	25%	62%	17%	[27%, 71%, 2%]
Among participants having four visits (n_4 = 120)							
Visit	[60, 69, 77]	[49, 61, 74]	[15, 26, 36]	22%	29%	10%	[38%, 58%, 4%]
Visit 2	[63, 72, 81]	[46, 62, 78]	[13, 25, 40]	22%	42%	11%	[38%, 58%, 4%]
Visit 3	[66, 75, 84]	[47, 61, 76]	[13, 26, 37]	18%	59%	16%	[38%, 59%, 3%]
Visit 4	[70, 78, 87]	[50, 63, 76]	[12, 25, 37]	17%	65%	18%	[38%, 60%, 2%]
Among participants having five visits (n_5 = 3)							
Visit 1	[62, 66, 66]	[49, 58, 67]	[18, 25, 33]	33%	33%	0%	[33%, 67%, 0%]
Visit 2	[65, 68, 70]	[59, 64, 70]	[18, 22, 26]	33%	33%	0%	[33%, 67%, 0%]
Visit 3	[68, 70, 72]	[16, 54, 68]	[18, 29, 78]	33%	0%	0%	[33%, 67%, 0%]
Visit 4	[71, 73, 74]	[14, 60, 67]	[17, 18, 83]	33%	0%	0%	[33%, 67%, 0%]
Visit 5	[73, 76, 77]	[15, 55, 75]	[13, 24, 82]	33%	33%	0%	[33%, 67%, 0%]

NAS, Normative Aging Study.

pack-years, daily alcohol consumption, and the number of years of education.

We obtained slightly greater estimates when we fitted the the generalized estimating equations (GEE) model for the association between sulfate and *ICAM-1* methylation, and between particle number, black carbon, and ozone and *IL-6* methylation. When we included censoring weights, our results were similar, except that we observed slightly stronger associations between ozone and methylation on *TLR-2* and *IL-6*.

Finally, the analysis restricted to days with 24-h $PM_{2.5}$ mass concentrations below 35 $\mu g/m^3$ showed no difference in significance and magnitude of our results, except that we obtained a significant association (over a monthly period) between sulfate and *ICAM-1* methylation. An interquartile range increase in sulfate concentration was associated with a 6% reduction in *ICAM-1* methylation (95% CI: -11% to 0%).

Discussion

We have previously reported positive associations between air pollution and levels of plasma fibrinogen, C-reactive protein (CRP), ICAM-1, and vascular cell adhesion protein 1 (VCAM-1) in this cohort; and our current results linking air pollution exposure to methylation on genes related to coagulation and inflammation pathways are consistent with those associations. We did not identify susceptible subgroups in which the association between air pollution and gene-specific methylation was stronger, except that we observed a greater effect of particle number on *IL-6* methylation in non-smokers compare with former and current smokers. The mediation analysis provided further insight into potential importance of the association between air pollution and gene-specific methylation because it suggests that the relationship between air pollution and protein levels are in part mediated by a change in gene-specific methylation.

Particle number and black carbon were negatively associated with *F3* methylation. Since hypomethylation is usually related to gene expression,[8] this result is consistent with higher fibrinogen levels observed after exposures to particle number and black carbon in the same cohort;[24] which is what the mediation analysis confirmed. F3, also known as tissue factor, is a key player in the coagulation cascade that results in fibrinogen production. Hypercoagulable states have been associated with high levels of tissue factor and fibrinogen.[25-27] *F3* expression and

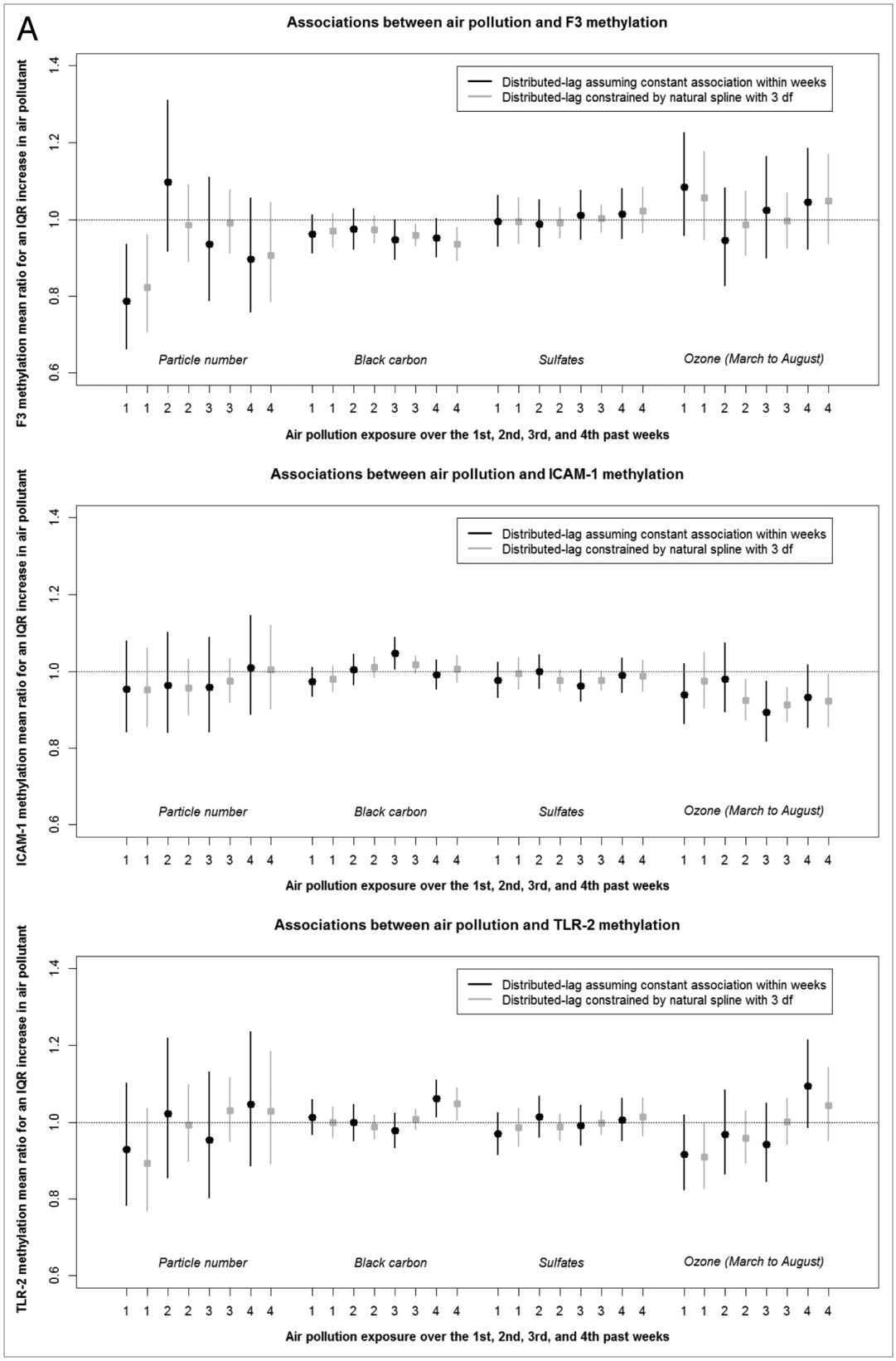

Figure 1A. Associations between air pollution and *F3*, *ICAM-1*, and *TLR-2* DNA methylation across the 1st to 4th weeks of exposure (estimates and associated 95% CI). Variables included in the models: f_1(air pollutant), f_2(temperature), f_3(relative humidity), age, body mass index, smoking status, diabetes status, statin use, percentage of neutrophils and lymphocytes in blood count, seasonal sine and cosine, season, and batch.

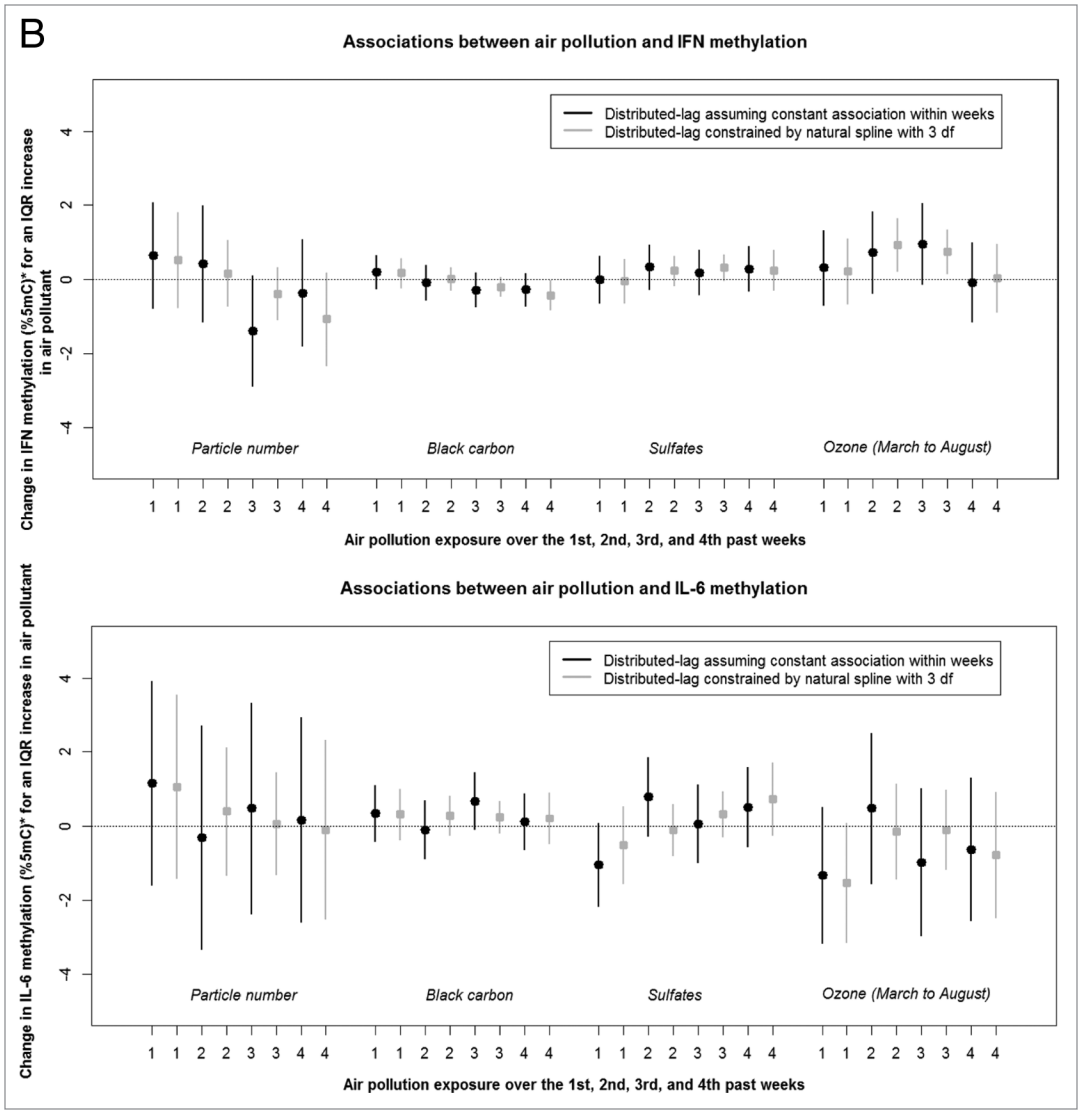

Figure 1B. Associations between air pollution and *IFN-γ*, and *IL-6* DNA methylation across the 1st to 4th weeks of exposure (estimates and associated 95% CI). *%5mC is the unit of DNA methylation and corresponds to the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines at position 5. Variables included in the models: f_1(air pollutant), f_2(temperature), and f_3(relative humidity) represent the distributed-lag functions with sets of coefficients constrained by a natural spline (with 3 degrees of freedom) that correspond to the air pollution, temperature, and relative humidity associations at lags 0 and 27 days.

fibrinogen have also been shown to increase in response to the intrusion of fine and ultrafine particles.[28-30] Exposure to fresh local and transported traffic emissions may therefore induce *F3* hypomethylation leading to increased tissue factor and fibrinogen production.

Similarly, we have previously shown that sulfate concentrations were associated with higher ICAM-1 protein levels in the same cohort.[24] In the present study, higher sulfate and ozone concentrations were related to *ICAM-1* hypomethylation. In our data, a 1%5mC decrease in *ICAM-1* methylation was associated with a 0.7% increase in ICAM-1 protein (95% CI: 0.0% to 1.4%). Our analyses of association and mediation suggested that exposure to sulfate and ozone decreased *ICAM-1* methylation, which could cause *ICAM-1* gene de-silencing and ICAM-1 protein overexpression.

In our secondary analysis across CpG positions, we observed a positive association between traffic-related pollutants and *IL-6* methylation and a negative association between ozone and *TLR-2* methylation. In the same cohort, *TLR-2* hypomethylation and *IL-6* hypermethylation were also associated with lower lung function[12] and ozone was positively related to CRP.[24] TLR-2, IL-6, and CRP are related to each other: they participate in innate immune responses and consist of early defense mechanisms against infections. A recent study linked TLR-2 to IL-6 when they observed greater air pollution associations on IL-6 for wild type mice compared with knockout mice for *TLR-2*.[31] High IL-6 levels, in turn, increase CRP.[32] We conclude that black carbon and ozone exposures may cause changes in *TLR-2* and *IL-6* methylation, which would be causing higher TLR-2, IL-6, and CRP levels. Our sensitivity analysis also suggests that

Table 2. Association between air pollution exposure over the 4-week period preceding medical examination and gene-specific DNA methylation

Methylation mean ratio for an interquartile range increase in air pollution								
	Particle number		Black carbon		Sulfate		Ozone (March to August)	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
F3	**0.725**	**0.655, 0.803**	**0.877**	**0.825, 0.932**	1.008	0.930, 1.093	1.081	0.885, 1.322
ICAM-1	**0.889**	**0.824, 0.961**	1.009	0.965, 1.055	0.948	0.896, 1.003	**0.759**	**0.664, 0.868**
TLR-2	0.943	0.851, 1.044	1.032	0.979, 1.088	0.987	0.920, 1.058	0.917	0.775, 1.086
Change in methylation (% 5mC) for an interquartile range increase in air pollution								
	Particle number		**Black carbon**		**Sulfate**		**Ozone (March to August)**	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
IFN-γ	−0.845	−1.704, 0.013	−0.363	−0.872, 0.145	0.589	−0.198, 1.376	**1.898**	**0.244, 3.552**
IL-6	1.412	−0.245, 3.068	0.806	−0.077, 1.688	0.317	−1.055, 1.688	−2.486	−5.424, 0.452

Variables included in the models: f_1(air pollutant)[b], f_2(temperature)[b], f_3(relative humidity)[b], age, body mass index, smoking status, diabetes status, statin use, % neutrophils in blood count, % lymphocytes in blood count, seasonal sine and cosine, season, and methylation batch. [b]f_1(air pollutant), f_2(temperature), and f_3(relative humidity) represent the distributed-lag functions with sets of coefficients constrained by a natural spline (with 3 degrees of freedom) that correspond to the air pollution, temperature, and relative humidity associations at lags 0 and 27 days.

air pollution affects methylation at specific positions within a gene's promoter region differently. Although taking the average methylation across specific position could give a more stable estimate of methylation on a certain gene's promoter region, this could be misleading.

We observed a positive association between ozone and *IFN-γ* methylation. Ozone exposure has been shown to increase IFN-γ protein levels.[33] IFN-γ is a cytokine that plays an important role in innate and adaptive immune responses against the intrusion of a foreign compound.[34] The direction of this association is also consistent with a previous study relating *IFN-γ* hypermethylation to lower lung function.[12] Ozone exposure could cause changes in *IFN-γ* methylation that would in turn regulate IFN-γ production.

Black carbon and particle number, which are associated with traffic emissions, were associated with *F3* and *IL-6* methylation, whereas sulfate and ozone, which are secondary pollutants that were transported to Boston, were related to changes in *ICAM-1*, *TLR-2*, and *IFN-γ* methylation. These findings suggest that air pollutants from distinct sources may affect intermediary cardiovascular-related blood markers differently. Even though our results do not prove that these methylation pathways are the primary reason for the associations between air pollution and plasma blood markers reported previously, they do suggest a plausible role in the exacerbation of cardiovascular morbidity and mortality due to air pollution.

Our analyses of effect modification did not reveal that participants' characteristics, such as smoking and obesity status, age, and baseline methylation levels of *LINE-1* and *Alu* elements identify subsets of individuals with stronger molecular responses to air pollution. We previously found in the same cohort that the association between air pollution and cardiovascular-related biomarkers was modified by methylation levels on *LINE-1* and *Alu* elements at baseline.[24] Cigarette smoking is known to modify DNA methylation levels.[35] Even though air pollution was related to gene-specific methylation in the overall sample containing never, current, and former smokers, we also found significant associations among never smokers.

Limitations and strengths

Our approach in this study is subject to some limitations. We did not compare our main findings to estimates obtained from a model that would multiply impute the missing values of the exposures, the covariates, and the outcomes. Although our assumption of Berkson measurement error for air pollution exposures assessed at a central site is supported by a previous study,[36] correction for measurement error may yield less biased estimates for spatially heterogeneous traffic-related air pollutants. In our analysis, we adjusted for potential confounding variables. We did not examine confounding due to other variables such as diet, although they are unlikely to be associated with intermediate-term air pollution. Our analysis is also limited to five genes. Methylation on other genes and other epigenetics mechanisms such as histone modifications and microRNA might be important variables to consider. We measured gene-specific methylation but not the levels of all associated proteins, which limit our power to detect effects in the mediation analysis. We measured levels of methylation and protein at the same visit. The potential for reverse causation is a limitation of our mediation findings. Potential risk factors or confounding variables included in our models, such as diabetes status and obesity, may also be intermediate variables. Our study population consists of white and elderly men and we cannot provide evidence for similar air pollution associations with gene-specific methylation in a different population.

Our approach has several strengths too. The prospective study design with repeated outcome measurements permitted us to perform a well-powered analysis. Our distributed-lag approach limited the number of tests and therefore the false positive rate, while assuring that complex lag structures are flexibly modeled. High precision pyrosequencing yields more

Table 3. Association between air pollution exposure over the 4-week period preceding medical examination on gene-specific DNA methylation according to the smoking status

Methylation mean ratio for an interquartile range increase in air pollution								
	Particle number		Black carbon		Sulfate		Ozone (March to August)	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
F3								
Never smokers	**0.739**	**0.648, 0.0.844**	**0.863**	**0.793, 0.939**	0.936	0.828, 1.058	1.032	0.773, 1.377
Former and current smokers	**0.719**	**0.644, 0.801**	**0.882**	**0.825, 0.943**	1.037	0.948, 1.135	1.106	0.882, 1.387
ICAM-1								
Never smokers	0.910	0.826, 1.002	1.005	0.943, 1.071	0.976	0.899, 1.059	**0.779**	**0.640, 0.948**
Former and current smokers	**0.880**	**0.810, 0.957**	1.011	0.964, 1.060	**0.933**	**0.877, 0.993**	**0.747**	**0.642, 0.868**
TLR-2								
Never smokers	0.911	0.800, 1.037	1.016	0.943, 1.094	0.939	0.848, 1.039	0.864	0.681, 1.098
Former and current smokers	0.958	0.859, 1.069	1.040	0.981, 1.102	1.007	0.932, 1.088	0.953	0.789, 1.152
Change in methylation (% 5mC) for an interquartile range increase in air pollution								
	Particle number		**Black carbon**		**Sulfate**		**Ozone (March to August)**	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
IFN-γ								
Never smokers	–0.805	–1.664, 0.053	**–0.651**	**–1.159, –0.143**	**1.197**	**0.411, 1.984**	**2.734**	**1.080, 4.388**
Former and current smokers	**–0.870**	**–1.729, –0.012**	–0.288	–0.796, 0.220	0.377	–0.410, 1.163	1.401	–0.253, 3.055
IL-6								
Never smokers	**2.877**	**1.221, 4.534**	0.553	–0.329, 1.436	–0.367	–1.738, 1.005	**–3.536**	**–6.474, –0.598**
Former and current smokers	0.886	–0.770, 2.543	**0.896**	**0.014, 1.779**	0.662	–0.710, 2.034	–2.028	–4.966, 0.910

Variables included in the models: f_1(air pollutant)[b], f_2(temperature)[b], f_3(relative humidity)[b], smoking status*f_1(air pollutant)[b], age, body mass index, smoking status, diabetes status, statin use, % neutrophils in blood count, % lymphocytes in blood count, seasonal sine and cosine, season, and methylation batch. f_1(air pollutant), f_2(temperature), and f_3(relative humidity) represent the distributed-lag functions with sets of coefficients constrained by a natural spline (with 3 degrees of freedom) that correspond to the air pollution, temperature, and relative humidity associations at lags 0 and 27 days.

accurate methylation measurements than are available from array data. DNA methylation constitutes a more stable biomarker than mRNA or protein expression, and therefore, represents a somewhat longer time window in biological responses. In addition, we checked for model misspecification by allowing the dose-response relationships between methylation and air pollution, temperature, and relative humidity to be non-linear. The similarity of the estimates we obtained using two different distributed-lag functions showed the robustness of our results. Our sensitivity analysis allowed us to identify positions within promoter regions of relevant genes that may play an important role in air pollution health effects. Our sensitivity analysis also presented stable results when we adjusted for additional potential confounding variables as well as when we considered GEE models with robust variance and censoring weights. The GEE estimator does not assume a normal distribution for the random intercepts and therefore provides more robust confidence intervals than the mixed-effects model, as well as population interpretation for non-Gaussian distributions. The directed acyclic diagram (DAG) we constructed also presented a setting with no time-varying confounding. Therefore, if our assumptions hold, the estimates we obtained by fitting association models should have a causal interpretation. The mediation analysis we performed allowed us to conclude that the air pollution effect on gene-specific methylation is likely to be followed by a change in cardiovascular-related proteins.

Materials and Methods

Study population

This prospective cohort study included participants from the Normative Aging Study, an investigation established in Boston in 1963 by the US. Veterans Administration.[24] We measured DNA methylation on blood samples collected after an overnight fast and smoking abstinence during the period 1999–2009. A total of 777 participants had their methylation assessed one

Table 4. Mediation analysis: the mediated effect of air pollution on cardiovascular-related biomarkers through a change in gene-specific methylation

Estimates correspond to a standard deviation increase from the mean				
	Particle number	Black carbon	Sulfate	Ozone (March to August)
	$\gamma_1\beta_2$ estimate [95% CI]	$\gamma_1\beta_2$ estimate [95% CI]	$\gamma_1\beta_2$ estimate [95% CI]	$\gamma_1\beta_2$ estimate [95% CI]
***F3* methylation and fibrinogen protein**	(–0.267)(–0.032) = 0.009 [–0.013, 0.030]	(–0.142)(–0.084) = 0.012 [0.000, 0.024]	(–0.054)(–0.099) = 0.005 [–0.003, 0.014]	(0.023)(–0.088) = –0.002 [–0.021, 0.017]
***ICAM-1* methylation and ICAM-1 protein**	(–0.053)(–0.077) = 0.004 [–0.004, 0.012]	(0.022)(–0.069) = –0.001 [–0.007, 0.004]	(-0.090)(–0.075) = 0.007 [0.000, 0.014]	(–0.441)(–0.085) = 0.037 [0.003, 0.072]
***TLR-2* methylation and CRP protein**	(–0.002)(0.023) = 0.000 [–0.002, 0.002]	(0.034)(–0.023) = –0.001 [–0.003, 0.002]	(–0.015)(–0.010) = 0.000 [–0.001, 0.001]	(–0.140)(–0.130) = 0.018 [–0.010, 0.047]
***IFN*-γ methylation and CRP protein**	(–0.101)(0.008) = –0.001 [–0.008, 0.006]	(–0.050)(0.039) = –0.002 [–0.006, 0.002]	(0.021)(0.017) = 0.000 [–0.001, 0.002]	(0.163)(0.050) = 0.008 [–0.006, 0.023]
***IL-6* methylation and IL-6 protein**	(0.037)(–0.071) = –0.003 [–0.009, 0.003]	(0.056)(–0.046) = –0.003 [–0.007, 0.001]	(0.003)(-0.046) = 0.000 [–0.003, 0.002]	(–0.128)(-0.024) = 0.003 [–0.008, 0.014]

Variables included in the models: air pollutant[b], temperature[b], relative humidity[b], age, body mass index, smoking status, diabetes status, statin use, % neutrophils in blood count, % lymphocytes in blood count, seasonal sine and cosine, season, and methylation batch. Air pollutant, temperature, and relative humidity represent the moving average between lags 0 and 27 days.

to five times with intervals of three to five years. We excluded observations with CRP levels over 10 mg/L so that the results are not confounded by infection state.[37] This study was approved by the institutional review boards of all participating institutions.

DNA methylation

We collected participant's blood at every visit and isolated DNA to assess gene-specific DNA methylation using highly quantitative methods based on bisulfite polymerase chain reaction pyrosequencing.[38] The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines at position 5 (%5mC).

We focused on genes that are expressed in blood leukocytes. We measured *F3*, *ICAM-1*, *TRL-2*, and *IFN*-γ methylation levels at two to five CpG positions within each gene's promoter region and calculated the mean values of the position-specific measurements. *IL-6* methylation was quantified outside the gene's promoter region. Exact positions within promoter regions have been previously described.[24]

Air pollution

The relevant exposure window for the air pollution association with methylation is unknown. Previous studies suggested an association spread over several weeks.[16,17,23] In a recent study, we observed weekly associations between air pollution and coagulation and inflammation markers in this cohort.[24] Therefore, we chose to explore a similar intermediate-term exposure window and focused on air pollution concentrations measured during the monthly period preceding each participant's methylation assessment.

Particulate concentrations were measured at the Harvard supersite located near downtown Boston and approximately 1 km from the examination center. Since the study participants lived in the Greater Boston area with a median distance of 20 km, we assumed that the ambient air pollutant concentrations could serve as surrogates of their exposures.

We measured hourly particle number per cm^3 (0.007–3 μm) with a Condensation Particle Counter (TSI Inc., Model 3022A) and hourly $PM_{2.5}$ black carbon concentrations using a Tapered Element Oscillation Microbalance (Model 1400A, Rupprecht and Pastashnick) and an Aethalometer (Magee Scientific Co., Model AE-16). We measured daily sulfate (SO_4^{2-}) concentrations with a Sulfate Particulate Analyzer (Thermo Electron Co., Model 5020) from 2000 to 2003. After this time period, SO_4^{2-} levels were estimated from elemental sulfur, measured by X-Ray Fluorescence analysis of Teflon filters. We did not measure ozone at the central monitoring site, but obtained hourly levels by averaging concentrations measured by two local state monitors operated by the Massachusetts Department of Environmental Protection.

Whereas particle number is a marker for fresh local traffic emissions, black carbon originates from both local and transported traffic emissions. Sulfates and ozone are secondary regional pollutants that were transported to Boston.

Statistical methods

Assumptions and directed acyclic graph (DAG)

Because we had repeated methylation measures for 71% of the participants, we fit generalized mixed-effects models with random intercepts to investigate whether air pollution levels weekly averaged over the 4-week period before the j^{th} visit of the i^{th} participant were associated with its methylation level at visit j (Y_{ij}). Because *F3*, *ICAM-1*, and *TLR-2* methylation had a point mass at zero and their residuals' distribution showed important deviation from a normal density, we assumed a Tweedie

distribution (**Fig. S1**) for these outcomes with a log-link and reported associations on the multiplicative scale. For *IFN-γ* and *IL-6* methylation, we assumed a Gaussian distribution for the regression residuals and presented our results on the additive scale.

We adjusted for potential confounders (C_1) such as: temperature, relative humidity, seasonal sine and cosine, season (Winter/Spring-Fall/Summer), and batch of methylation measurement. We also controlled for factors likely to influence methylation (C_2) such as: age, race, diabetes, body mass index, smoking status, statin use, as well as percentages of neutrophils and lymphocytes in differential blood count. We included C_2 in the models for efficiency and blocking any potential back-door path through unmeasured variables that would be a common cause of air pollution and C_2.[39] We thus assumed no unmeasured confounding between air pollution and methylation, given the random intercept and the C_1 and C_2 vectors (**Fig. 2**). Moreover, we assumed the missing mechanisms of the exposures and outcomes to be at random conditional on the covariates, and the air pollution measurement error to be primarily Berkson.

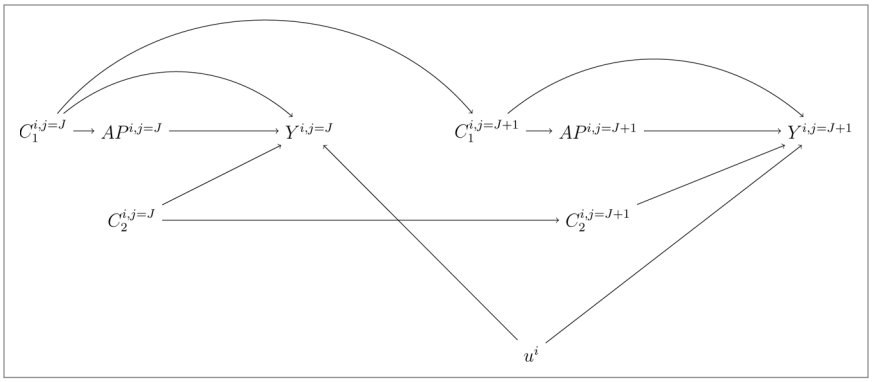

Figure 2. Directed acyclic graph (DAG) at visits j = J and j = J+1. We present the directed acyclic graph (DAG) at adjacent visits j = J and j = J+1 to illustrate the relationships we assumed between the variables included in the regression models. $AP^{i,j}$ represents 1st, 2nd, 3rd, and 4th weeks averages of air pollutant concentration before the j^{th} visit of the i^{th} participant. $Y^{i,j}$ represents the i^{th} participant gene-specific DNA methylation at visit j. $C_1^{i,j}$ and $C_2^{i,j}$ corresponds to the potential confounding variables and risk factors of DNA methylation for participant i^{th} at visit j, respectively. u^i represents the random intercept of participant i.

We checked for non-linear dose-response relationships between methylation and air pollution, temperature, and relative humidity using generalized additive models and cubic splines. We found no deviation from linear dose-response relationships with respect to methylation. We explored the nature of the air pollution association with methylation over time using distributed-lag linear models (lags 0 to 27 days) and examined how the association between the methylation outcomes and lagged exposure changes across lags. This methodology, previously developed for the analysis of time series data,[40] is extended here in the context of individual longitudinal data. We chose natural splines with three degrees of freedom to model the non-linear shape of the distributed-lag. Because the association between methylation and air pollution exposure varied over the exposure lags we examined, we calculated from the distributed-lag model cumulative associations over four weekly periods (lags 0 to 6, 7 to 13, 14 to 20, and 21 to 27 days) preceding the j^{th} visit of the i^{th} participant, as well as cumulative associations over the entire monthly period.

Main regression models

Distributed-lag model for *F3*, *ICAM-1*, and *TLR-2* (multiplicative scale)

$$\log E[Y_{ij}] = (\gamma_0+u_i)+f_1(\gamma_1,AP_{ij})+f_2(\gamma_2,\text{Temperature}_{ij})+ f_3(\gamma_3,\text{Relative humidity}_{ij})+\Sigma_k\,\gamma_{4k}\,C_{1kij}+\Sigma_k\,\gamma_{5k}\,C_{2kij}$$

with Y_{ij}~Tweedie and $u_i \sim N(0,\sigma_u^2)$

Distributed-lag linear models for *IFN-γ* and *IL-6* (additive scale)

$$Y_{ij} = (\gamma_0+u_i)+f_1(\gamma_1,AP_{ij})+f_2(\gamma_2,\text{Temperature}_{ij})+ f_3(\gamma_3,\text{Relative humidity}_{ij})+\Sigma_k\,\gamma_{4k}\,C_{1kij}+\Sigma_k\,\gamma_{5k}\,C_{2kij}+\varepsilon_{ij}$$

with $\varepsilon_{ij} \sim N(0,\sigma^2)$ and $u_i \sim N(0,\sigma_u^2)$

f_1, f_2, and f_3 represent distributed-lag functions with sets of coefficients γ_1, γ_2, and γ_3 constrained by a natural spline (with 3 degrees of freedom) that correspond to the air pollution, temperature, and relative humidity associations with methylation at lags 0 to 27 days. C_1 and C_2 represent sets of variables for which we adjusted.

Analyses of effect modification and mediation

In order to detect any differences in the relationship between air pollution and gene-specific methylation in susceptible subgroups, we used interaction terms to examine whether the association between air pollution and methylation differed according to participants' smoking status, obesity status, age, as well as baseline levels of *LINE-1* and *Alu* methylation.

We also conducted mediation analyses and calculated the mediated effect of air pollution (AP_{ij}) on relevant inflammatory and coagulation markers (Y_{ij}) through a change in DNA methylation (M_{ij}). We fit simultaneously two linear mixed-effects models:[41]

$$M_{ij} = (\gamma_0+u_i)+\gamma_1 AP_{ij}+\Sigma_k\,\gamma_{2k}\,C_{1kij}+\Sigma_k\,\gamma_{3k}\,C_{2kij}+\varepsilon_{ij}$$

with $\varepsilon_{ij} \sim N(0,\sigma^2)$ and $u_i \sim N(0,\sigma_u^2)$

$$Y_{ij} = (\beta_0+g_{0i})+\beta_1 AP_{ij}+(\beta_2+g_{2i})\,M_{ij}+\Sigma_k\,\beta_{3k}\,C_{1kij}+\Sigma_k\,\beta_{4k}\,C_{2kij}+\eta_{ij}$$

with $\eta_{ij} \sim N(0,\sigma^2)$, $g_{0i} \sim N(0,\sigma_{0u}^2)$, and $g_{2i} \sim N(0,\sigma_{2u}^2)$

$\boldsymbol{C_1}$ and $\boldsymbol{C_2}$ represent sets of variables for which we adjusted. The mediated effect is given by the product formula $\gamma_1\beta_2$. The delta method allowed us to approximate the variance of the mediated effect by $\text{Var}(\beta_2)\,\gamma_1^2 + 2\,\text{Cov}(\gamma_1,\beta_2)\,\gamma_1\,\beta_2 + \text{Var}(\gamma_1)\beta_2^2$.

Sensitivity analyses

We considered another choice of distributed-lag functions that assume constant lag association within weeks. This assumption is equivalent to fitting a model that simultaneously includes four consecutive weekly moving averages of air pollution, temperature, and relative humidity. For simplicity and to limit the number of tests, our additional secondary analyses examined only cumulative associations over the entire monthly period.

Our main analysis considered the average methylation of CpG sites in each gene's promoter region. However, methylation

at specific positions within a gene's promoter region may affect gene expression differently. Therefore, we examined whether the air pollution associations we observed in the main analysis were specific to certain positions within the gene's promoter region. Exact positions within promoter region have been described in a previous paper.[24]

Because of the negative correlations between ozone and the traffic-related air pollutants (particle number and black carbon) as well as between sulfate and particle number in our data, we also fit co-pollutant models including either ozone and particle number, ozone and black carbon, or sulfate and particle number. To confirm that our results were not subject to residual confounding, we further adjusted for cigarette pack-years, more than two alcohol drinks a day, and years of education. We also checked the robustness of the confidence intervals obtained in the main analysis by fitting generalized estimating equations (GEE) allowing an exchangeable correlation structure and a sandwich variance. In addition, we further explored the effect of censoring due to incomplete follow-up by using inverse probability censoring weights (IPCW) in the same GEE framework. The construction of the censoring weights has already been described.[12] This methodology assumes that participants with complete and incomplete follow-up are exchangeable, conditional on the covariates. The probability of each participant to providing complete data at all visits is first estimated. The analysis is then restricted to the complete data and each participant is assigned a weight inversely proportional to his estimated probability of complete follow-up. We finally excluded observations for which the 24 h $PM_{2.5}$ mass concentrations exceeded the daily standard of 35 $\mu g/m^3$ and examined the robustness of our results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/27584

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