Rapid DNA Methylation Changes after Exposure to Traffic Particles

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***Rationale*: Exposure to particulate air pollution has been related to increased hospitalization and death, particularly from cardiovascular disease. Lower blood DNA methylation content is found in processes related to cardiovascular outcomes, such as oxidative stress, aging, and atherosclerosis.**

***Objectives*: We evaluated whether particulate pollution modifies DNA methylation in heavily methylated sequences with high representation throughout the human genome.**

***Methods*: We measured DNA methylation of long interspersed nucleotide element (LINE)-1 and Alu repetitive elements by quantitative polymerase chain reaction–pyrosequencing of 1,097 blood samples from 718 elderly participants in the Boston area Normative Aging Study. We used covariate-adjusted mixed models to account for within-subject correlation in repeated measures. We estimated the effects on DNA methylation of ambient particulate pollutants (black carbon, particulate matter with aerodynamic diameter $\leq$ 2.5 μm [$PM_{2.5}$], or sulfate) in multiple time windows (4 h to 7 d) before the examination. We estimated standardized regression coefficients (β) expressing the fraction of a standard deviation change in DNA methylation associated with a standard deviation increase in exposure.**

***Measurements and Main Results*: Repetitive element DNA methylation varied in association with time-related variables, such as day of the week and season. LINE-1 methylation decreased after recent exposure to higher black carbon ($\beta = -0.11$; 95% confidence interval [CI], -0.18 to -0.04; $P = 0.002$) and $PM_{2.5}$ ($\beta = -0.13$; 95% CI, -0.19 to -0.06; $P < 0.001$ for the 7-d moving average). In two-pollutant models, only black carbon, a tracer of traffic particles, was significantly associated with LINE-1 methylation ($\beta = -0.09$; 95% CI, -0.17 to -0.01; $P = 0.03$). No association was found with Alu methylation ($P > 0.12$).**

***Conclusions*: We found decreased repeated-element methylation after exposure to traffic particles. Whether decreased methylation mediates exposure-related health effects remains to be determined.**

Keywords: epigenetic processes; air pollution; inhalation exposure; interspersed repetitive sequences

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Changes in epigenetic markers, such as DNA methylation, may mediate environmental effects on human health. Short-term exposure to traffic pollution has been linked with cardiovascular diseases, which are characterized by a loss of blood DNA methylation.

What This Study Adds to the Field

Our data show that blood leukocyte DNA methylation in sequences with widespread genomic representation decreases rapidly following peaks of higher ambient levels of traffic particles. These changes may contribute to produce environmental effects on human health.

DNA methylation is a mechanism of epigenetic regulation that, in mammals, involves the addition of methyl groups to cytosine to form 5-methylcytosine. About 55% of the human genome consists of repetitive elements, including approximately 500,000 long interspersed nucleotide elements (LINE-1, also indicated as L1) and 1.4 million Alu repetitive elements, which are heavily methylated (1). Demethylation of LINE-1 and Alu elements increase their activity as retrotransposable sequences, which may induce genomic alterations by insertion and or homologous recombination (2), and deregulate gene transcription (3). Repetitive elements are activated during conditions of cellular stress, and LINE-1 expression has been recently identified as a mediator of ischemic heart damage (4). Because of their high representation throughout the genome, LINE-1 and Alu methylation has been shown to correlate with global genomic DNA methylation content (1, 5). Although changes in DNA methylation have been proposed to mediate the effects of environmental factors on human health (6), relations with environmental exposures and the time-related patterns of such variations are largely unexplored.

Epidemiologic time-series studies have identified an association between daily changes in concentration of ambient air pollution and daily number of deaths and hospitalizations, particularly from cardiovascular disease and after relatively short timelags after exposure peaks (7, 8). It has been suggested that traffic-derived particles are the most toxic component (9), and the ambient level of black carbon particles, used as a tracer for traffic pollution, has been consistently associated with a variety of adverse health outcomes (10–14). Processes related to cardiovascular disease, such as oxidative stress (15), atherosclerosis (16), and aging (17), have been associated with lower DNA methylation content in blood DNA. Initial observations from *in vitro* and animal models have shown that air particles,

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particularly those from traffic emissions such as black carbon or diesel exhaust (18), or metal components of air particles such as arsenic and cadmium (19) can induce changes in gene-specific or global DNA methylation. In human studies, particulate air pollution exposure, including exposure to black carbon and particulate matter ≤2.5 μm in aerodynamic diameter ($PM_{2.5}$), has been related to hyperhomocysteinemia (14, 20), a marker of decreased availability of methyl-group donors that is associated with decreased global DNA methylation content (16). In addition, subjects with lower methyl-nutrient intakes or defective polymorphisms in the methyl-nutrient pathway have been recently found to be more susceptible to the negative effects of particulate pollution on heart rate variability (21). Whether exposure to particulate air pollution induces changes in DNA methylation in human subjects has not been investigated.

In the present study, we determined the effect of ambient particulate matter levels on blood DNA methylation in LINE-1 and Alu repetitive elements and the time-related patterns of DNA methylation variations after the exposure. Some of the results of this study were presented at the American Thoracic Society International Conference, Toronto 2008 (Publication page A266) and at the International Society for Environmental Epidemiology 2008 Annual Conference (Abstract #837).

METHODS

Study Subjects

The study included 718 elderly individuals (mean age, 73.3 yr; range, 55–100 yr) evaluated between January 1999 and June 2007 as part of the Normative Aging Study, a longitudinal investigation of aging established in 1963 by the US Veterans Administration. The participants of the Normative Aging Study are all male subjects who have reported for medical examination every 3 to 5 years. A blood sample was collected at each visit. During the study period, 368 subjects reported for examination only one time, 321 reported two times, and 29 reported three times, for a total of 1,097 samples collected. This study was approved by the Institutional Review Boards of all participating institutions. All participants gave written informed consent.

Air Pollution Measurement

Ambient $PM_{2.5}$, black carbon, and sulfate were measured at a stationary monitoring site 1 km from the examination site. Sulfate concentrations were not measured between October 1, 2004 and December 31, 2006. Therefore, the total number of observations with available sulfate data was 671 (61%). We evaluated different time windows of exposure, using as exposure index the average pollutant concentrations taken from time periods of 4 hours to 7 days (moving averages) before the time of blood draw because these exposure windows have shown the strongest association with air pollution health effects in previous studies (22–24). Distributions of the average ambient pollutant levels are reported in Table 1.

Laboratory Methods

Methods for DNA methylation analysis have been previously described (25) and are reported in detail in the online supplement. Briefly, we performed DNA methylation analysis of Alu and LINE-1 repetitive elements on bisulfite-treated blood leukocyte DNA using highly quantitative polymerase chain reaction (PCR)–pyrosequencing technology. The degree of methylation was expressed for each DNA locus as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Each marker was tested in three replicates, and their average was used in the statistical analysis.

Statistical Analysis

Because our study included repeated measures of DNA methylation for many participants, the data are correlated. To deal with this, we fitted mixed effects models (PROC MIXED in SAS V9.0), assuming:

$$Y_{it} = b_0 + u_i + b_1 X_{1it} + \cdots + b_p X_{pit} + \beta \text{Pollution}_i + \varepsilon_{it}$$

TABLE 1. DISTRIBUTION OF THE AVERAGE LEVEL OF AMBIENT PARTICULATE POLLUTANTS WITHIN 4 HOURS TO 7 DAYS BEFORE THE EXAM

			Percentile				
Moving Average	Mean	SD	10th	25th	50th	75th	90th
Black carbon							
4-h mean	1.35	0.90	0.47	0.68	1.10	1.70	2.61
1-d mean	0.89	0.46	0.39	0.53	0.79	1.17	1.56
2-d mean	0.85	0.39	0.40	0.55	0.77	1.12	1.41
3-d mean	0.79	0.33	0.40	0.52	0.73	1.01	1.27
4-d mean	0.76	0.29	0.41	0.54	0.72	0.94	1.15
5-d mean	0.76	0.26	0.44	0.56	0.73	0.94	1.10
6-d mean	0.78	0.25	0.48	0.60	0.76	0.95	1.10
7-d mean	0.79	0.24	0.48	0.62	0.78	0.98	1.12
$PM_{2.5}$							
4-h mean	12.2	7.7	5.1	6.7	10.3	15.3	22.8
1-d mean	10.9	6.3	5.0	6.5	9.3	13.6	19.5
2-d mean	10.6	5.2	5.3	6.9	9.5	13.1	17.2
3-d mean	10.4	4.8	5.7	7.0	9.3	12.8	16.3
4-d mean	10.3	4.3	5.99	7.3	9.5	12.3	15.5
5-d mean	10.3	3.9	6.3	7.7	9.7	12.1	14.9
6-d mean	10.3	3.5	6.7	7.8	9.8	12.2	14.5
7-d mean	10.3	3.3	6.9	8.0	9.8	12.0	14.3
Sulfate*							
4-h mean†	NA	NA	NA	NA	NA	NA	NA
1-d mean	3.24	2.79	0.88	1.50	2.53	4.02	5.67
2-d mean	3.12	2.21	1.03	1.67	2.67	3.85	5.75
3-d mean	3.01	1.84	1.22	1.86	2.56	3.62	5.23
4-d mean	2.95	1.58	1.38	1.97	2.55	3.62	4.86
5-d mean	2.93	1.40	1.47	1.98	2.62	3.60	4.75
6-d mean	2.98	1.30	1.64	2.12	2.73	3.58	4.72
7-d mean	2.98	1.24	1.70	2.19	2.77	3.51	4.57

Definition of abbreviation: $PM_{2.5}$=particulate matter ≤2.5 μm in aerodynamic diameter.

* Sulfate ambient measurements were not available between October 1, 2004 and December 31, 2006. The total number of observations with available sulfate data was 671 (61%)

† Sulfate data were taken as 24-h integrated measurements before October 1, 2004. Consequently, 4-h moving averages were not computed.

where Y_{it} is the DNA methylation level in either LINE-1 or Alu in subject *i* at time *t*, b_0 is the overall intercept, u_i is the separate random intercept for subject *i*, and X_{1it}—X_{pit} are the p covariates for subject i at each time t. We evaluated the association between particle levels and DNA methylation in multivariable models adjusted for age, body mass index, cigarette smoking (never, former, current), pack-years, statin use, fasting blood glucose, diabetes mellitus, percent lymphocytes and neutrophils in differential blood count, day of the week, season, and outdoor temperature. We reported standardized regression coefficients (β) expressing the fraction of a standard deviation change in LINE-1 or Alu methylation associated with a standard deviation increase in particle level. All tests were two tailed. A P value of < 0.05 was considered statistically significant. All analyses were performed in SAS 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

The general characteristics of the study participants are shown in Table 2. Mean blood DNA methylation levels, expressed as the percentage of 5-methylcytosine (%5mC), were 77.2 (SD = 2.2) for LINE-1 and 26.2 (SD = 1.2) for Alu.

We first explored whether DNA methylation varied in association with time-related variables that might have been operating as confounders in the analysis of air pollution effects (Table 3). In unadjusted regression models, LINE-1 methylation was associated with day of the week (on exam days) and season. LINE-1 methylation was lowest in blood DNA samples taken on Mondays (Reference) and showed the highest levels on Wednesdays (β = 0.35; 95% confidence interval [CI], 0.10–0.59; P = 0.006). Exam days in the Normative Aging Study are Monday through Thursday; therefore, methylation data were

TABLE 2. CHARACTERISTICS OF THE STUDY POPULATION IN THE NORMATIVE AGING STUDY

Variable	All visits (*n = 1,097*)	Visit #1 (*n = 718*)	Visit #2 (*n = 350*)	Visit #3 (*n = 29*)
Age, yr (SD)	73.3 (6.7)	72.3 (6.8)	75.0 (6.1)	75.9 (5.2)
Body mass index, kg/m^2 (SD)	28.2 (4.1)	28.2 (4.0)	28.0 (4.1)	29.3 (4.7)
Smoking status, n (%)				
Never smoker	338 (31)	214 (30)	115 (33)	9 (31)
Former smoker	714 (65)	476 (66)	219 (62)	19 (65)
Current smoker	45 (4)	28 (4)	16 (5)	1 (4)
Cumulative smoking,* pack-years (SD)	30.8 (26.7)	31.4 (27.7)	29.5 (24.3)	31.6 (30.4)
Fasting blood glucose, mg/dL (SD)	108.5 (26.6)	109.5 (28.8)	106.4 (21.6)	109.8 (22.4)
Diabetes, n (%)	215 (20)	138 (19)	71 (20)	6 (21)
Treatment with statins, n (%)	434 (40)	246 (34)	176 (50)	12 (41)
Blood count				
White blood cells, cells/mm^3 (SD)	6,638 (2,955)	6,605 (3,322)	6,670 (2,002)	7,093 (2,947)
Neutrophils, % (SD)	62.2 (8.7)	62.1 (8.6)	62.5 (9.0)	61.6 (8.0)
Lymphocytes, % (SD)	25.5 (8.0)	25.6 (7.9)	25.2 (8.3)	26.4 (7.7)
Monocytes, % (SD)	8.6 (2.1)	8.7 (2.1)	8.5 (2.2)	8.6 (2.4)
Basophils, % (SD)	0.6 (0.5)	0.6 (0.5)	0.6 (0.5)	0.8 (0.5)
Eosinophils, % (SD)	3.3 (2.1)	3.3 (2.1)	3.4 (2.2)	3.1 (2.1)
Day of the visit, n (%)				
Monday	69 (6)	69 (10)	0 (0)	0 (0)
Tuesday	244 (22)	152 (21)	86 (25)	0 (0)
Wednesday	580 (53)	321 (45)	236 (67)	6 (21)
Thursday	204 (19)	176 (24)	28 (8)	23 (79)
Season of the visit, n (%)				
Spring	286 (26)	180 (25)	93 (27)	13 (45)
Summer	300 (27)	203 (28)	94 (27)	3 (10)
Fall	319 (29)	210 (29)	109 (31)	0 (0)
Winter	192 (18)	125 (17)	54 (15)	13 (45)
Outdoor temperature, °C (SD)	12.8 (9.2)	13.1 (8.8)	12.6 (9.8)	5.8 (10.6)
DNA methylation, %5mC† (SD)				
LINE-1	77.2 (2.2)	76.9 (1.9)	77.5 (2.1)	82.7 (1.8)
Alu	26.2 (1.2)	26.3 (1.1)	26.2 (1.2)	24.6 (1.2)

Definition of abbreviation: LINE-1 = long interspersed nucleotide elements.

* Mean and SD among ever-smokers.

† Percentage of 5-methylcytosine.

not available for the other days of the week. LINE-1 methylation levels were highest in spring ($\beta = 0.32$; 95% CI, 0.16–0.49; $P < 0.0001$) and lowest in fall (Reference). Alu methylation showed a seasonal pattern opposite to that observed for LINE-1, with the lowest levels in spring ($\beta = -0.26$; 95% CI, −0.41 to −0.10; $P = 0.002$) and highest in fall (Reference), and had no significant association with day of the week. Outdoor temperature showed weak, nonsignificant associations with LINE-1 and Alu methylation (Table 3).

LINE-1 methylation decreased in relation to higher black carbon and $PM_{2.5}$ ambient level (Table 4), with significant associations for all the time windows evaluated and stronger effects at the longer time windows (2–7 d). Sulfate levels showed negative but not significant associations with LINE-1 methylation. Alu methylation showed no significant association with pollutant levels at any of the time windows evaluated (Table 4). To further investigate the effects of black carbon and $PM_{2.5}$ on LINE-1 methylation, we ran models including the 4-hour and 7-day moving averages. In these models (Table 5), only the 7-day moving averages showed an independent effect on LINE-1 methylation.

Using the 7-day moving averages, we fit two-pollutant models that showed an independent significant effect of black carbon on LINE-1 methylation ($\beta = -0.09$; 95% CI, −0.17 to

TABLE 3. ASSOCIATION OF LINE-1 AND Alu METHYLATION WITH DAY OF THE WEEK, SEASON, AND OUTDOOR TEMPERATURE

	Effect on LINE-1 Methylation*		Effect on Alu Methylation*	
Variable	Beta (95% CI)	*P* Value	Beta (95% CI)	*P* Value
Day of the week				
Monday	Reference		Reference	
Tuesday	0.04 (−0.22 to 0.30)	0.77	0.04 (−0.22 to 0.30)	0.77
Wednesday	0.35 (0.10 to 0.59)	0.006	−0.17 (−0.41 to 0.07)	0.17
Thursday	0.23 (−0.04 to 0.50)	0.10	0.00 (−0.27 to 0.26)	0.97
Season				
Fall	Reference		Reference	
Winter	0.16 (−0.03 to 0.34)	0.093	−0.05 (−0.23 to 0.13)	0.56
Spring	0.32 (0.16 to 0.49)	<0.0001	−0.26 (−0.41 to −0.10)	0.002
Summer	0.08 (−0.07 to 0.24)	0.29	−0.05 (−0.21 to 0.10)	0.49
Temperature	−0.04 (−0.10 to 0.02)	0.21	0.05 (−0.01 to 0.11)	0.09

Definition of abbreviations: CI = confidence interval; LINE-1 = long interspersed nucleotide elements.

* Unadjusted standardized regression coefficients and 95% CIs expressing the fraction of a standard deviation change in LINE-1 or Alu methylation associated with a specific category of day of the week and season or a standard deviation increase in temperature.

TABLE 4. EFFECTS OF AMBIENT LEVELS OF BLACK CARBON, $PM_{2.5}$, AND SULFATE ON LINE-1 AND Alu METHYLATION

Moving Average	Effects on LINE-1 Methylation		Effect on Alu Methylation*	
	Coeff* (95% CI)	*P* Value	Coeff* (95% CI)	*P* Value
Black carbon				
4-h mean	−0.07 (−0.13 to −0.01)	0.03	−0.02 (−0.08 to 0.04)	0.50
1-d mean	−0.09 (−0.15 to −0.02)	0.007	−0.02 (−0.08 to 0.05)	0.58
2-d mean	−0.10 (−0.16 to −0.03)	0.004	−0.02 (−0.09 to 0.05)	0.56
3-d mean	−0.10 (−0.16 to −0.03)	0.005	−0.02 (−0.09 to 0.05)	0.62
4-d mean	−0.09 (−0.15 to −0.02)	0.01	−0.01 (−0.08 to 0.06)	0.80
5-d mean	−0.09 (−0.15 to −0.02)	0.0089	0.01 (−0.05 to 0.08)	0.73
6-d mean	−0.10 (−0.17 to −0.04)	0.002	0.01 (−0.06 to 0.07)	0.87
7-d mean	−0.11 (−0.18 to −0.04)	0.002	0.01 (−0.06 to 0.08)	0.75
$PM_{2.5}$				
4-h mean	−0.07 (−0.13 to −0.01)	0.03	0.03 (−0.03 to 0.09)	0.28
1-d mean	−0.09 (−0.16 to −0.02)	0.008	−0.01 (−0.07 to 0.05)	0.74
2-d mean	−0.10 (−0.17 to −0.03)	0.003	−0.01 (−0.07 to 0.05)	0.82
3-d mean	−0.10 (−0.17 to −0.04)	0.003	−0.01 (−0.07 to 0.05)	0.78
4-d mean	−0.10 (−0.16 to −0.03)	0.004	−0.01 (−0.07 to 0.05)	0.75
5-d mean	−0.10 (−0.16 to −0.03)	0.004	−0.01 (−0.07 to 0.05)	0.84
6-d mean	−0.11 (−0.17 to −0.04)	0.001	−0.01 (−0.07 to 0.05)	0.74
7-d mean	−0.13 (−0.19 to −0.06)	<0.001	−0.01 (−0.07 to 0.05)	0.71
Sulfate†				
4-h mean‡	NA	NA	NA	NA
1-d mean	−0.08 (−0.16 to 0.01)	0.07	0.02 (−0.06 to 0.10)	0.66
2-d mean	−0.07 (−0.15 to 0.02)	0.12	0.07 (−0.02 to 0.15)	0.13
3-d mean	−0.07 (−0.15 to 0.02)	0.11	0.06 (−0.02 to 0.15)	0.16
4-d mean	−0.06 (−0.15 to 0.03)	0.19	0.07 (−0.02 to 0.15)	0.14
5-d mean	−0.06 (−0.15 to 0.02)	0.14	0.07 (−0.02 to 0.15)	0.12
6-d mean	−0.04 (−0.12 to 0.04)	0.33	0.06 (−0.02 to 0.14)	0.16
7-d mean	−0.04 (−0.12 to 0.04)	0.36	0.05 (−0.03 to 0.13)	0.25

Definition of abbreviations: CI = confidence interval; LINE-1 = long interspersed nucleotide elements; $PM_{2.5}$=particulate matter with aerodynamic diameter ≤2.5 μm.

* Standardized correlation coefficients expressing the fraction of a standard deviation change in DNA methylation associated with a standard deviation change in pollutant level adjusted for age, body mass index, cigarette smoking (never, former, current), pack-years, statin use, fasting blood glucose, diabetes mellitus, percent lymphocytes, and neutrophils in differential blood count, day of the week, season, and outdoor temperature.

† Sulfate ambient measurements were not available between October 1, 2004 and December 31, 2006. The total number of observations with available sulfate data was 671 (61.1%)

‡ Sulfate data were taken as 24-h integrated measurements before October 1, 2004. Consequently, 4-h moving averages were were not computed.

−0.01; $P = 0.03$), whereas the association between $PM_{2.5}$ and LINE-1 methylation was not statistically significant (β = −0.03; 95% CI, −0.11 to 0.04; $P = 0.43$).

Because these results showed that air particle levels are time-varying determinants of LINE-1 methylation, we fit multivariable models to adjust for particulate levels the association of day of the week and season with LINE-1 methylation. In models adjusting for the 7-day moving average of black carbon level, day of the week ($P \geq 0.11$) and season ($P \geq 0.18$) were not significantly associated with LINE-1 methylation.

As a sensitivity analysis, we tested for nonlinear effects of ambient pollutants on LINE-1 methylation in a set of models in which air particle levels were fitted using nonlinear terms (i.e, penalized splines with degrees of freedom varying from 1 to 10). Our results showed that none of the models with penalized splines had a better fit for pollutant levels compared with the linear models, suggesting that the decreases in LINE-1 methylation associated with the exposure were linear.

DISCUSSION

Prior studies have linked particulate air pollution with oxidative stress production, activation of inflammatory pathways, endothelial injury and dysfunction, arterial vasoconstriction, and alterations in blood coagulation (26–28). However, the nature of the intermediate changes linking particulate air pollution to cardiovascular disease is not fully understood (8). In the present investigation on a large population of elderly individuals, we found that blood DNA methylation in repetitive elements was decreased in individuals with recent exposure to higher levels of ambient particulate matter. In particular, LINE-1 methylation was decreased in association with ambient levels of black carbon and $PM_{2.5}$ measured in the 7 days before the examination,

TABLE 5. EFFECTS OF BLACK CARBON AND $PM_{2.5}$ ON LINE-1 METHYLATION IN MODELS INCLUDING SHORT-TERM (4-h) AND MID-TERM (7-d) MOVING AVERAGES OF AMBIENT POLLUTANT LEVEL

	Effects on LINE-1 Methylation	
	Coeff* (95% CI)	*P* Value
Black carbon (4-h + 7-d model)		
4-h mean	−0.05 (−0.11 to 0.02)	0.16
7-d mean	−0.09 (−0.16 to −0.02)	0.008
$PM_{2.5}$ (4-h + 7-d model)		
4-h mean	−0.04 (−0.11 to 0.03)	0.24
7-d mean	−0.11 (−0.18 to −0.05)	0.001

For definition of abbreviations, *see* Table 4.

* Standardized correlation coefficients, expressing the fraction of a standard deviation change in DNA methylation associated with a standard deviation change in pollutant level, adjusted for age, body mass index, cigarette smoking (never, former, current), pack-years, statin use, fasting blood glucose, diabetes mellitus, percent lymphocytes and neutrophils in differential blood count, day of the week, season, and outdoor temperature.

whereas no association was observed between Alu methylation and particle levels.

Previous *in vitro* experiments have shown that repetitive element hypomethylation and transcription occur in response to biological processes, such as cellular stress and inflammation (4), which are also induced by particulate pollution in exposed subjects (8, 26, 27). Air particles are known to increase the production of reactive oxygen species, perhaps in a catalytic fashion via redox cycling (27). Oxidative DNA damage can interfere with the ability of methyltransferases to interact with DNA (15), thus resulting in hypomethylation of cytosine residues at CpG sites. In addition, reactive oxygen species have been recently shown to alter the expression of genes belonging to DNA methylation machinery (29). Changes in DNA methylation through generation of reactive oxygen species may be induced by components of airborne particulate matter, such as metals (19). Takiguchi and colleagues showed that cadmium interferes with DNA methylation by inhibiting DNA methyltransferases, possibly due to an interaction of cadmium with the methyltransferase DNA binding domain (30). In addition, depletion of the cellular methyl pool has been indicated as a contributor to the global hypomethylation observed in arsenic-treated cells (19). Hypomethylation of repetitive DNA sequences is expected to lead to the transcriptional activation of those repetitive sequences that still contain active promoters, potentially resulting in disruption of transcription factor balance, sense or antisense transcriptional interference, and production of transcripts complementary to endogenous transcripts or to alterations in genomic organization and stability (2, 3). The decrease in methylation we observed may be part of the systemic events consequent to alveolar inflammation and the release of inflammatory mediators resulting from particle inhalation (27). Also, inhaled nanoparticles may move out of the lung into the bloodstream (8), where they may directly affect blood cells by inducing oxidative stress or inflammatory reactions (8, 15, 16).

In our study, the association of black carbon and $PM_{2.5}$ with LINE-1 methylation was significant for all the time windows we examined, which included the average of hourly concentrations measured over 4 hour to 7 days before the examination. We selected those time windows because short-term health effects of air particle exposure, including increased numbers of hospitalizations and deaths resulting from cardiovascular disease, have been found to correlate with higher ambient pollutant levels measured within 1 to 7 days before the outcome (23), and 5- to 7-day cumulative exposures have been associated with outcomes related to cardiovascular disease, such as increased serum fibrinogen (24, 31) and C-reactive protein (26). The time patterns of changes in DNA methylation are largely unknown. In patients with myeloid leukemia treated with the demethylating analog 5-Aza-2′-deoxycytidine, LINE-1 methylation has been shown to decrease starting 2 to 4 days after the beginning of the treatment (32). Our data show that environmental exposures may operate on DNA methylation over a similar time frame, with effects from particles that were significant even in association with the shorter time windows evaluated (4 hours). However, when we fit models that included the shortest (4-h) and longest (7-d) moving averages that we used in our analyses, only the 7-day effects remained significant. This finding suggests that particles may need several days to operate changes on DNA methylation. Black carbon is a tracer of particles from traffic, which are a major component of fine particles measured by $PM_{2.5}$. The finding that only the black carbon effect on LINE-1 methylation was statistically significant in two-pollutant models including black carbon and $PM_{2.5}$ indicates traffic particles as the main determinant of the observed changes in DNA methylation. In these models, the standardized regression coefficient for the 7-day moving average of black carbon was equal to -0.09, indicating that a standard deviation increase in black carbon resulted in an estimated decrease in LINE-1 methylation equal to 9% of its standard deviation. To put this result into perspective, this decrease in LINE-1 methylation can be compared with the results of a recent analysis in this same study that showed that LINE-1 methylation decreases progressively with age (33). The effect on LINE-1 methylation determined by a standard deviation increase in black carbon concentration would be equivalent in size to a decline in LINE-1 methylation occurring over 3.4 years. In other words, assuming that a decrease in LINE-1 methylation reflects biological age (17), the black carbon effect would be similar to increased aging of nearly 3.5 years. However, whether this LINE-1 methylation decline is associated with increased risk of age-related diseases—including the increased risk of cardiovascular disease associated with air pollution exposure (7, 8)—remains to be determined. Also, experimental studies in humans or *in vitro* and animal models are warranted to confirm the exposure–methylation link and to clarify the epigenetic mechanisms of the action of traffic particles. Associations of LINE-1 methylation with sulfate, an indicator of coal combustion particles, never achieved statistical significance, although missing data gave that analysis less power.

In our data, we did not observe any correlation of Alu methylation with particle levels. Although LINE-1 and Alu repetitive element methylation have been shown to correlate with genomic DNA methylation content, the two repetitive elements are controlled through different mechanisms and have been shown to have different transcription patterns in response to cellular stressors (33). In addition, although our study had the advantage of high-precision quantitative Pyrosequencing measures, the lower availability of nonmutated CpG sites in Alu elements may have resulted in lower sensitivity, compared with LINE-1 methylation measures, to detect potential changes in global DNA methylation (1).

By examining potential confounders of the exposure-methylation relation, we observed differences in LINE-1 and Alu DNA methylation by season and day of the week, which we then controlled in multivariable models testing for particle effects. Epigenetic adaptive changes to season and temperature have been reported in plants and aquatic species (34, 35). In our data, LINE-1 and Alu methylation had associations with mean outdoor temperature of the day of the exam that, although not significant, were consistent with those observed with season. A previous study of 30 healthy volunteers reported cyclic variations in blood DNA methylation content observed over a 24-hour period (36), and recent investigations have shown transient cyclical methylation and demethylation of specific gene promoters (37, 38). The association of LINE-1 methylation with day of the week observed in our data may provide further evidence for the existence of cyclical patterns of variation in repetitive element DNA methylation. However, our analyses of temporal variations in DNA methylation were performed to identify potential confounders of air pollution effects because levels of air pollutants, particularly those from traffic emissions, show strong variations in association with day of the week and season (14, 21, 23). In multivariable models adjusting for air particle levels, the associations of day of the week and season with LINE-1 methylation were not statistically significant, suggesting that the cyclical variations in LINE-1 methylation we observed are dependent, at least in part, on changes in particle levels. Our study was not designed to evaluate temporal cycles of DNA methylation and provides only descriptive, nonconclusive evidence for the existence of such patterns, which

should be confirmed in studies evaluating DNA methylation in multiple repeated measures taken in different days and seasons.

Human exposure to combustion particles is predominantly from traffic particles (39). In urban areas black carbon, which we used as a tracer for particles from traffic emissions, derives primarily from exhaust emissions from diesel-powered vehicles (40). Because black carbon stems from incomplete combustion, which usually contains toxic substances, it is considered a better indicator of the health effect of air particles than the weight measurement (11). A potential limitation of this study is that we used ambient black carbon levels, as well as levels of $PM_{2.5}$ and sulfate, from a single monitoring site as a surrogate for recent exposure to air particles. $PM_{2.5}$ and sulfate concentrations in Boston are very spatially homogeneous (14), so it is reasonable to conclude that $PM_{2.5}$ and sulfate from a single monitoring site could characterize personal exposures to them. By contrast, black carbon concentration is considerably more spatially variable, depending on traffic exposure. However, a recent study comparing ambient concentrations at this site with 14 monitoring stations in metropolitan Boston ranging from downtown to rural showed correlation coefficients varying from 0.54 to 0.94 (median, 0.80) (14). Although these results suggest that some degree of misclassification is possible, due to the nondifferential nature of the error, this would be expected to bias the results toward the null. Our results can only be generalized to an aged population that consists of older men who are almost all white. The effect on women and children and on different ethnic groups should be addressed in future studies, particularly in relation to the exposure of different population groups with varying geographical location, occupation, socioeconomic status, and behavioral characteristics. Our study was based on a quantitative analysis of blood DNA methylation using pyrosequencing, which is highly reproducible and accurate for measuring small changes in DNA methylation (1, 25), and was repeated three times on each sample to minimize the assay variability. Whether the changes we observed in blood DNA reflect similar modifications of DNA methylation in tissues directly exposed to particles, such as the airway epithelium or different target tissues, and whether the amount of changes we observed is associated with negative health effects need to be assessed in future studies.

In conclusion, our results indicate that repetitive element methylation in blood DNA, particularly in LINE-1 sequences, varies in relation to environmental and time variables, including significant decreases after short-term exposure to air particles from traffic emissions. Our data indicate the existence of rapid methylation changes *in vivo* that may trigger adaptive responses and produce environmental effects on human health. Whether the alteration of these molecular mechanisms predicts the effects of traffic particles on human health remains to be determined.

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