Online Data Supplement

Rapid DNA Methylation Changes after Exposure to Traffic Particles

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Laboratory Methods for DNA Methylation Analysis

Blood Collection: 7 ml of whole blood were collected by venous phlebotomy in EDTA tubes. Buffy coat was extracted and stored in cell lyses solution until DNA extraction. All samples were coded and frozen at –20°C.

Bisulfite treatment: DNA was extracted from stored frozen buffy coat of 7 mL whole blood, using the QiAmp DNA blood kits (QIAGEN). 1μg DNA (concentration 50 ng/μl) was treated using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 μl of M-Elution Buffer. Bisulfite-treated DNA was stored at –20°C until use.

PCR and pyrosequencing: DNA methylation was quantitated using bisulfite-PCR and Pyrosequencing (E1, E2). In brief, the samples were bisulfite-treated and PCR-amplified (see Table E1 at the end of this supplement for PCR primers and conditions). A biotin-labeled primer was used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham-Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing Inc., Westborough, MA), as per manufacturer's recommendations. Then, 0.3 μM pyrosequencing primer was annealed to the purified single-stranded PCR product and pyrosequencing was performed using the Pyromark MA System (Pyrosequencing Inc., Westborough, MA). The degree of methylation was expressed for each DNA locus as % methylated cytosines over the sum of methylated and unmethylated cytosines. We used non-CpG cytosine residues as built-in controls to verify bisulfite conversion. Each marker was tested in three replicates and their average was used in the statistical analysis. LINE-1 and Alu element PCR was used for Pyrosequencing-based methylation analysis using previously published methods (E2), with the following modifications. A 50 μl PCR was carried out in 25 μl of GoTaq Green Master mix (Promega, Madison, WI, USA), 1 pmol

biotinylated forward primer, 1 pmol reverse primer, 50 ng bisulfite-treated genomic DNA and water.

Exposure assessment

Measurement of Ambient Pollutant Levels

Ambient $PM_{2.5}$, black carbon, and sulfate were measured with a Tapered Element Oscillating Microbalance (TEOM; model 1400A, Rupprecht & Pataschnick Co, Albany, NY), aethalometer (Magee Scientific, Berkeley, Calif), and Harvard-EPA annular denuder system sampler, respectively. $PM_{2.5}$ and BC were measured continuously, with data processed as one-hour averages. Sulfate concentrations were measured as 24-h averaged concentrations. Standard quality assurance and control procedures were used for collecting, processing and reporting all measurements, following EPA established procedures (EPA Quality Assurance Handbook for Air Pollution Measurement Systems, 1998).

The initial analysis of air pollution data included univariate explorations of all variables, using histograms, statistical summaries, and other graphical techniques. Expected ranges for all of the variables was defined a priori, and out of range values, or values identified as outliers were checked for errors. Outliers that appeared to be real were kept in the data sets, but the Multivariate Outlier Detection Algorithm (E3) was used to eliminate outliers in all variables before constructing the final analytical datasets. While these observations were real, we eliminated outliers in exposure because they have extremely high leverage and may dominate regression results. This approach eliminates the possibility that any associations seen are driven by a few extreme observations.

Calculation of moving averages

To evaluate the association between particle exposure and DNA methylation, we calculated moving averages to reflect the exposure in the hours/days before the examination. We averaged hourly measurements of air pollution recorded in the *x* hours before the time of examination, where *x* was a number ranging from 4 (4-hour moving average) to 168 (7-day moving average, i.e., 168 hours). In regression models, we used the moving averages for 4 hours, and 1, 2, 3, 4, 5, 6, or 7 days. Each moving average was computed only if at least 75% of the hourly measurements were present.

Statistical Analysis: Model Selection

Most of the dependent variables included in the models (i.e., age, body mass index, cigarette smoking, fasting blood glucose, diabetes, day of the week, season, statin use, outdoor temperature) were selected based on previous regression analyses we used to evaluate the effects of particulate pollutants on cardiovascular endpoints such as heart rate variability (E4-E6). In the present work, we included additional dependent variables for the following reasons: i) pack-years of smoking were added to provide further control for smoking habits, given that some previous reports have associated tobacco smoking to DNA hypomethylation (E7); ii) percent lymphocyte and neutrophils counts were added because, as DNA methylation was measured on unfractionated whole blood, shifts in white blood cell counts induced by the exposure might have spuriously affected the results. In univariate analysis, LINE-1 methylation was significantly associated with percent lymphocytes (beta=-0.10; 95% CI -0.17, -0.04; p=0.001) and neutrophils (beta=0.08; 95% CI 0.02, 0.15; p=0.01). In contrast, Alu methylation was associated with neither percent lymphocytes (beta=-0.04; 95% CI -0.10, 0.02; p=0.021), nor percent neutrophils (beta=0.03; 95% CI -0.03, 0.09; p=0.01).

In our models, LINE-1 and Alu methylation were modeled in their original scale. We also ran an additional set of models using the logarithm of LINE-1 or Alu methylation. Distribution of LINE-1 and Alu methylation was moderately asymmetric on the original scale, and approximation to normal distribution was slightly better on the log scale. However, the results of the models using log

methylation differed only marginally from those using the original scale, and statistical significance was not affected. Therefore, results from models with LINE-1/Alu on the original scale are presented throughout the paper.

We used mixed regression models to account for within-subject correlation in repeated measures obtained from the same subjects. The models, however, also included individuals with only one measure, and were thus based on all the individuals with available DNA methylation and exposure data.

ADDITIONAL REFERENCES FOR ONLINE DATA SUPPLEMENT

E1. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite pcr of repetitive DNA elements. *Nucleic Acids Res* 2004;32:e38.

E2. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, Byun HM, Jiang J, Marinelli B, Pesatori AC, Bertazzi PA, Yang AS. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res* 2007;67:876-880.

E3. Rosner B. Percentage points for a generalized esd many-outlier procedure. *Technometrics* 1983;25:165-172.

E4. Baccarelli A, Cassano PA, Litonjua A, Park SK, Suh H, Sparrow D, Vokonas P, Schwartz J. Cardiac autonomic dysfunction: Effects from particulate air pollution and protection by dietary methyl nutrients and metabolic polymorphisms. *Circulation* 2008;117.

E5. Chahine T, Baccarelli A, Litonjua A, Wright RO, Suh H, Gold DR, Sparrow D, Vokonas P, Schwartz J. Particulate air pollution, oxidative stress genes, and heart rate variability in an elderly cohort. *Environ Health Perspect* 2007;115:1617-1622.

E6. Schwartz J, Park SK, O'Neill M S, Vokonas PS, Sparrow D, Weiss S, Kelsey K. Glutathione-s-transferase m1, obesity, statins, and autonomic effects of particles: Gene-by-drug-by-environment interaction. *Am J Respir Crit Care Med* 2005;172:1529-1533.

E7. Ting Hsiung D, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, Kelsey KT. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev* 2007;16:108-114.

Table E1

Primers and PCR conditions for DNA methylation analyses

	LINE-1 assay	Alu assay
Forward primer (5' to 3')	TTTTGAGTTAGGTGTGGGATATA	Biotin-TTTTTATTAAAAATATAAAAATT
Reverse primer (5' to 3')	Biotin-AAAATCAAAAAATTCCCTTTC	CCCAAACTAAAATACAATAA
Sequencing primer (5' to 3')	AGTTAGGTGTGGGATATAGT	AATAACTAAAATTACAAAC
PCR conditions	95°C for 30 s, 50°C for 30 s, 72°C for 30 s (35 cycles)	96°C for 90 s, 43°C for 60 s, 72°C for 120 s (40 cycles)