Particulate matter, the newborn methylome, and
cardio-respiratory health outcomes in childhood

Carrie V. Breton¹,*, Lu Gao¹, Jin Yao¹, Kimberly D. Siegmund¹, Fred Lurmann², and Frank Gilliland¹

¹University of Southern California, Dept of Preventive Medicine, 2001 N Soto St, Los Angeles, CA 90089, USA; and ²Sonoma Technology Inc, 1455 N. McDowell Blvd, Suite D, Petaluma, CA 94954-6503, USA

*Correspondence address: Carrie Breton, ScD., Department of Preventive Medicine, USC Keck School of Medicine, 2001 N. Soto Street, Los Angeles, CA 90032, USA. Tel: +1 (323) 442-7383; Fax: +1 (323) 442-3272; E-mail: breton@usc.edu

Abstract

Ambient air pollution is associated with adverse health outcomes including cardio-respiratory diseases. Epigenetic mechanisms such as DNA methylation may play a role in driving such associations. We investigated the effects of prenatal particulate matter (PM) exposure on DNA methylation of 178,309 promoter regions in 240 newborns using the Infinium HumanMethylation450 BeadChip, using a generalized linear regression model with a quasi-binomial link family, adjusted for gender, plate, and cell types. PM-associated CpG loci were then investigated for their associations with childhood asthma, carotid intima-media thickness (CIMT), and blood pressure (BP) using logistic or linear regression. Thirty-one loci were associated with either PM₁₀ or PM₂·₅ using FDR-corrected p-values of less than 0.15. Two loci were evaluated for replication in a separate population of 280 Children’s Health Study (CHS) subjects using Pyrosequencing, of which one successfully replicated (COLEC11 cg03579365). Three of the 31 loci were also associated with physician-diagnosed asthma at 6 years old, two were associated with CIMT and one with systolic BP at 10 years old. A higher methylation level in TM9SF2 (cg02015529) and UBE2S (cg00035623), respectively, was associated with a 2SD increase in prenatal PM and was also associated with 36% and 98% increased odds of asthma; whereas methylation of TDRD6 (cg22329831) was negatively associated with PM and a 24% decreased odds of asthma. Prenatal PM exposure was associated with altered DNA methylation in newborn blood in a small number of gene promoters, some of which were also associated with cardio-respiratory health outcomes later in childhood.

Keywords: methylation, particulate matter, air pollution, asthma, cardiovascular

Introduction

Ambient air pollution is associated with numerous adverse health outcomes, the most notable of which are allergic, respiratory, and cardiovascular diseases [1–3]. Long-term exposures have been associated with measures of atherosclerosis, including carotid intima-media thickness (CIMT) and blood pressure, both of which predict future cardiovascular events [4–8]. Regional and near-roadway pollutants have also been associated with childhood asthma, but the strength of association may depend on timing and duration of exposures [9–11]. Air pollutant exposures early in life, particularly during the prenatal period, have been associated with asthma development by age...
6 years in some but not all studies [12, 13]. Prenatal air pollutant exposures have not been evaluated for their contribution to cardiovascular disease (CVD) risk, although this hypothesis is supported by the developmental origins of adult disease theory (DOHaD) and increasing data from animal models [14–22].

The biological mechanisms driving these exposure-disease associations are thought to be largely through oxidative stress, inflammation, or endothelial or autonomic dysfunction [23]. Importantly, genetic variation in genes involved in these biological pathways can alter an individual’s susceptibility to air pollution health effects [24], providing further evidence for their involvement. In recent years, the hypothesis that epigenetics might play a role in driving exposure-disease associations has gained traction, in part, because epigenetic modifications are labile and may respond to environmental exposures in ways that directly or indirectly affect gene transcription and disease risk.

Several studies have now been conducted to evaluate the effects of air pollutants on epigenetics, with a primary focus on DNA methylation [25]. Particulate matter (PM) for example, has been associated with hypomethylation of selected tandem repeats [26] as well as changes in DNA methylation in a handful of candidate genes involved in asthma, inflammation, and oxidative stress [27–31]. These studies have been conducted in adults and largely in occupational or controlled exposure settings over relatively short exposure time windows. Moreover, few studies have taken an epigenome-wide approach in evaluating exposure effects.

Given our current understanding of the important and dynamic role that DNA methylation plays in embryogenesis [32] and the likelihood that epigenetic mechanisms play a role in the DOHaD hypothesis [19], we sought to investigate the effects of prenatal particulate matter (PM$_{10}$ and PM$_{2.5}$) exposure on DNA methylation profiles in newborns using the Infinium HumanMethylation450 BeadChip (HM450) in a subset of the Children’s Health Study [33, 34]. PM-associated CpG loci were also investigated for their associations with childhood cardiorespiratory health outcomes, including asthma prevalence, carotid intima-media thickness, and systolic and diastolic blood pressure.

**Results**

Demographic characteristics of the 240 study subjects at study entry as well as the 280 subjects in the replication population are shown in Table 1. Generally the characteristics of both populations were similar. Participants averaged 11 years of age (range 10–13 years) at the time of CIMT assessment, although there were more females (58%) than males (42%) in the study population. Sixteen (7%) were exposed to maternal smoking during pregnancy, while 29 (12%) were exposed to paternal smoking. The replication population had no subjects exposed to maternal smoking. Prevalence of asthma was 10–12%.

Distributions of prenatal air pollutant exposures and cardiovascular phenotypes are shown in Figure 1 and Table 1. The median levels of PM$_{10}$ were 38.9, 41.1, 39.2 μg/m$^3$ for the first, second, and third trimesters, respectively, with an interquartile range 19.3, 20.6, and 17.8 μg/m$^3$ whereas median levels of PM$_{2.5}$ were 26.3, 26.7, and 24.0 μg/m$^3$ with an interquartile range of 8.1, 8.2, and 5.6 μg/m$^3$, respectively. PM$_{10}$ and PM$_{2.5}$ were modestly correlated within each trimester, but levels were not correlated across trimesters ($r^2$ ranged from 0.54 to 0.69) (see online Supplementary material, Table S1).

The effects of trimester specific PM$_{10}$ and PM$_{2.5}$ on methylation of 178 309 promoter CpG loci were evaluated. We identified one CpG (cg22506605) associated with second trimester PM$_{10}$ (FDR-corrected P-value = 0.07) and nine CpGs associated with third trimester PM$_{10}$ (FDR-corrected P-value < 0.15) (Table 2). We also identified 10 CpGs associated with second trimester PM$_{2.5}$ and 11 CpGs associated with third trimester PM$_{2.5}$ using FDR-corrected P-values of less than 0.15 (Table 3). One CpG was found to be associated with both PM$_{2.5}$ and PM$_{10}$. The locus cg17486097 in the UNCSD gene was associated with higher methylation for both third trimester PM$_{10}$ and PM$_{2.5}$. Additional analyses evaluating potential confounding of these associations by maternal education, in utero tobacco smoke exposure and ethnicity largely did not change the results (see online Supplementary material, Tables S2 and S3).

We chose two loci with the largest magnitude of effects from each analysis for further replication: cg03579365 in the COLEC11 gene from the PM$_{10}$ analysis and cg27277978 in the SEFH52 gene from the PM$_{2.5}$ analysis (Figure 2). Replication was conducted in newborn bloodspots from an additional population of 280 subjects selected from the Children’s Health Study [33, 34] using Pyrosequencing. Replication for cg03579365 was successful (Table 4), further supporting an association between methylation in this locus and PM$_{10}$; however, the results were strongest for first trimester rather than third trimester exposures. Replication was not successful for cg27277978.

Finally, we related methylation level at birth in the list of PM-associated CpG loci with cardiovascular and respiratory health outcomes in early childhood. Of the 31 loci tested, three were associated with physician-diagnosed asthma at 6 years of age, two were associated with CIMT and one with systolic BP z-score at 11 years of age (Table 5). A higher methylation level in the promoters of TM9SF2 (cg02015529) and UBE2S (cg00035623), respectively, was associated with a 2SD increase in prenatal PM and was also associated with 36 and 98% increased odds of asthma at 6 years of age; whereas methylation of TDRD6 (cg22329831) was negatively associated with PM and a 24% decreased odds of asthma.

**Discussion**

Evidence demonstrating associations between air pollution and DNA methylation is sparse and few, if any, studies have evaluated epigenome-wide DNA methylation at birth in association with prenatal exposures. The epidemiologic studies that do exist have been conducted in adults and largely in occupational or controlled exposure settings over relatively short exposure time windows. In these studies, particulate matter has been associated with hypomethylation of selected tandem repeats [26] as well as changes in DNA methylation in a handful of candidate genes involved in asthma, inflammation, and oxidative stress [27–31, 35–37]. In vitro studies in murine macrophages have also provided evidence that PM$_{2.5}$ alters methylation machinery, specifically by decreasing expression levels of DNMTs [38]. In one of the only studies to evaluate prenatal PM exposure, Janssen et al. found that prenatal PM$_{2.5}$ was associated with mitochondrial DNA methylation in placental tissue [39, 40].

In this study, we found that prenatal exposure to PM$_{10}$ and PM$_{2.5}$ was associated with altered DNA methylation in newborn blood in a small number of gene promoters, some of which were also associated with cardio-respiratory health outcomes later in childhood. The loci associated with either PM$_{2.5}$ or PM$_{2.5}$ were largely independent, with only one locus associated with both exposures. The association between PM exposure and one locus in the COLEC11 gene promoter was replicated in an independent population of subjects using a second laboratory
Table 1: demographic characteristics and cardio-respiratory phenotypes of participants

|                               | Primary study population (N = 240) | Replication population (N = 280) |
|-------------------------------|------------------------------------|----------------------------------|
|                               | N       | %      | Median | Min | Max | IQR    | N       | %      | Median | Min | Max | IQR    |
| Male sex                      | 100     | 41.7   | –      | –   | –   | –      | 140     | 50.0   | –      | –   | –   | –      |
| Race/Ethnicity                |         |        |        |     |     |        |         |        |        |     |     |        |
| Hispanic White                | 137     | 57.1   | –      | –   | –   | –      | 151     | 53.9   | –      | –   | –   | –      |
| Non-Hispanic White            | 73      | 30.4   | –      | –   | –   | –      | 93      | 33.2   | –      | –   | –   | –      |
| Asian                         | 15      | 6.3    | –      | –   | –   | –      | 11      | 3.9    | –      | –   | –   | –      |
| Black/Other                   | 15      | 6.3    | –      | –   | –   | –      | 25      | 8.9    | –      | –   | –   | –      |
| Maternal smoking during pregnancy | 16      | 6.7    | –      | –   | –   | –      | 0       | 0.0    | –      | –   | –   | –      |
| Paternal smoking during pregnancy | 29      | 12.0   | –      | –   | –   | –      | 28      | 10.0   | –      | –   | –   | –      |
| Asthma                        | 23      | 9.6    | –      | –   | –   | –      | 33      | 11.8   | –      | –   | –   | –      |
| Mother’s Education            |         |        |        |     |     |        |         |        |        |     |     |        |
| High school or less           | 76      | 31.7   | –      | –   | –   | –      | 80      | 28.6   | –      | –   | –   | –      |
| Some college                  | 79      | 32.9   | –      | –   | –   | –      | 96      | 34.3   | –      | –   | –   | –      |
| College grad/some grad school | 69      | 28.8   | –      | –   | –   | –      | 86      | 30.7   | –      | –   | –   | –      |
| Age at CIMT assessment        | –       | –      | 11.2   | 9.9 | 12.8 | 1.0    | –       | –      | 11.3   | 10.0| 12.8| 1.0    |
| CIMT, μm                      | –       | –      | 564.5  | 398.8| 674.2| 48.9    | –       | –      | 565.3  | 434.5| 692.5| 55.0    |
| BMI, kg/m²                    | –       | –      | 19.2   | 13.6| 34.2 | 5.5    | –       | –      | 18.6   | 13.6| 34.9 | 5.4    |
| SBP, mmHg                     | –       | –      | 105.0  | 85.0| 129.0| 13.0    | –       | –      | 104.0  | 86.0| 124.0| 11.3    |
| DBP, mmHg                     | –       | –      | 56.0   | 46.0| 80.0 | 7.5     | –       | –      | 56.7   | 40.3| 83.7 | 7.3     |

CIMT – carotid intima-media thickness, BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure.

Numbers do not always add up to 100% due to missing data.

Table 2: Association between a 2SD increase in prenatal PM₁₀ exposure and DNA methylation (n = 240)**.

| Probe            | Chr | Position | Gene         | Mean methylation | Coef | P-value | Coef | P-value | Coef | P-value | Direction of effect across trimesters |
|------------------|-----|----------|--------------|------------------|------|---------|------|---------|------|---------|--------------------------------------|
| cg22506605       | 20  | 44462172 | SNX21        | 0.06             | 0.06 | 0.15    | 0.20 | 3.7 × 10⁻⁷* | 0.04 | 0.32    | +++                                  |
| cg17466097       | 8   | 35093411 | UNC5D        | 0.02             | −0.02| 0.40    | 0.06 | 3.4 × 10⁻³ | 0.11 | 2.7 × 10⁻⁷* | −++                                  |
| cg22329831       | 6   | 46655820 | TDRD6        | 0.95             | 0.00 | 0.96    | −0.10| 2.4 × 10⁻² | −0.19| 1.0 × 10⁻⁶* | −++                                  |
| cg02015529       | 13  | 10015320 | TM8SF2       | 0.04             | −0.03| 0.67    | 0.18 | 9.9 × 10⁻⁴ | 0.24 | 5.4 × 10⁻⁶* | −++                                  |
| cg03579365       | 2   | 3642211  | COLEC11      | 0.89             | 0.02 | 0.50    | 0.08 | 0.01     | 0.14 | 4.5 × 10⁻⁶* | +++                                  |
| cg04048259       | 20  | 57875346 | EDN3         | 0.02             | 0.05 | 0.24    | 0.09 | 0.03     | 0.17 | 4.1 × 10⁻⁶* | +++                                  |
| cg07575624       | 1   | 19277862 | RGS2         | 0.05             | 0.01 | 0.82    | 0.06 | 0.23     | 0.20 | 4.2 × 10⁻⁶* | +++                                  |
| cg13394864       | 2   | 97563733 | FAM178B      | 0.78             | 0.00 | 0.82    | 0.00 | 0.85     | −0.06| 5.9 × 10⁻⁶* | −++                                  |
| cg15155738       | 12  | 12145433 | C12orf43     | 0.01             | −0.04| 0.19    | −0.08| 4.6 × 10⁻³| −0.13| 5.4 × 10⁻⁶* | −−                                   |
| cg25430696       | 17  | 2240014  | TSR1;SGSM2   | 0.07             | −0.03| 0.32    | 0.10 | 3.9 × 10⁻⁴| 0.15 | 2.1 × 10⁻⁸* | −−++                                 |

*Indicates loci that are statistically significant after FDR correction (<0.15).

**Results are from a quasi-binomial regression model adjusted for gender, plate, and cell types. 2 SD for PM₁₀ for trimesters 1, 2, and 3 are 32.4, 32.6, and 31.6.

Figure 1: (a–b) distribution of cumulative air pollutant exposures across three trimesters (n = 240). (a) PM₂.₅ and (b) PM₁₀.
Table 3: Association between a 2SD increase in prenatal PM2.5 exposure and DNA methylation (n = 185)**.

| Probe       | Chr | Position | Gene  | Trimester 1 | Trimester 2 | Trimester 3 |
|-------------|-----|----------|-------|-------------|-------------|-------------|
|             |     |          |       | Mean methylation | Coeff | P-value | Coeff | P-value | Coeff | P-value | Coeff | P-value | Direction of effect |
| cg01439366  | 20  | 388553   | RBCK1 | 0.04        | -0.01 | 0.62    | 0.13 | $5 \times 10^{-6}$ | *   | 0.03 | 0.27    | -     | ++        |
| cg02733795  | 6   | 33422526 | ZBTB9 | 0.04        | 0.06  | 0.07    | 0.14 | $2.8 \times 10^{-6}$ | *   | 0.08 | 0.02    | ++    | ++        |
| cg04335339  | 10  | 116853118| ATRNL1| 0.05        | 0.02  | 0.43    | 0.13 | $2.9 \times 10^{-7}$ | *   | -0.04 | 0.18    | ++    | ++        |
| cg04784263  | 16  | 57318624 | PLIP  | 0.02        | 0.07  | 0.06    | 0.17 | $5.1 \times 10^{-7}$ | *   | 0.03 | 0.36    | +++   | ++        |
| cg05233674  | 6   | 34433773 | PACSIN1| 0.03        | 0.07  | 0.22    | 0.22 | $5.0 \times 10^{-6}$ | *   | 0.02 | 0.79    | +++   | +         |
| cg06618764  | 7   | 2719090  | AMZ1  | 0.96        | -0.06 | 0.72    | -0.59 | $5.4 \times 10^{-6}$ | *   | -0.13 | 0.42    | -      | -         |
| cg10109500  | 3   | 172165884| GHSR  | 0.03        | 0.00  | 0.95    | 0.12 | $7.5 \times 10^{-6}$ | *   | -0.01 | 0.65    | 0       | +         |
| cg16522462  | 20  | 1875565  | SIRPA | 0.03        | 0.04  | 0.17    | 0.12 | $6.5 \times 10^{-6}$ | *   | -0.05 | 0.07    | +++   | +         |
| cg20909686  | 11  | 65554042 | OVOL1 | 0.04        | -0.01 | 0.88    | 0.20 | $4.5 \times 10^{-6}$ | *   | -0.05 | 0.32    | -      | -         |
| cg27399414  | 1   | 9884593  | CLSTN1| 0.02        | 0.07  | 0.10    | 0.18 | $8.4 \times 10^{-6}$ | *   | -0.01 | 0.74    | ++    | +         |
| cg00035623  | 19  | 55919836 | UBE2S | 0.04        | -0.01 | 0.73    | 0.02 | 0.68     | 0.18 | 6.5 $\times 10^{-7}$ | *   | -      | ++        |
| cg02656441  | 13  | 111367915| ING1  | 0.05        | -0.10 | 0.12    | 0.09 | 0.17     | 0.27 | 1.3 $\times 10^{-6}$ | *   | -      | ++        |
| cg07246225  | 2   | 145277659| ZEB2  | 0.06        | -0.01 | 0.87    | -0.04 | 0.61     | 0.30 | 5.0 $\times 10^{-6}$ | *   | -      | -         |
| cg10217503  | 12  | 65153209 | GNS   | 0.02        | -0.06 | 0.08    | 0.01 | 0.70     | 0.17 | 1.7 $\times 10^{-6}$ | *   | -      | ++        |
| cg16252178  | 12  | 98909434 | TMPO2 | 0.04        | -0.03 | 0.31    | -0.03 | 0.32     | 0.17 | 1.4 $\times 10^{-6}$ | *   | -      | ++        |

*Indicates loci that are statistically significant after FDR correction (<0.15).

**Results are from a quasi-binomial regression model adjusted for gender, plate, and cell types. 2 SD for PM2.5 for trimesters 1, 2, and 3 are 13.8, 14.2, and 11.2.

Figure 2: Scatterplot of 3rd trimester PM$_{10}$ exposure and methylation values in cg2727798 (SEPHS2) and cg03579365 (COLEC11).

Table 4: Effects of per 2SD increase in prenatal PM$_{10}$ or PM$_{2.5}$ exposure on DNA methylation in a replication population*.

| Pollutant | Probe       | Gene     | Trimester 1 | Trimester 2 | Trimester 3 |
|-----------|-------------|----------|-------------|-------------|-------------|
|           |             | Mean methylation | N | $\beta$ | P-value | % change in methylation | $\beta$ | P-value | % change in methylation | $\beta$ | P-value | % change in methylation |
| PM$_{10}$ | cg03579365  | COLEC11  | 0.91 | 0.08 | 0.02 | 0.62% | -0.008 | 0.81 | -0.06% | -0.05 | 0.14 | -0.42% |
| PM$_{2.5}$| cg27277978 | SEPHS2   | 0.02 | 0.14 | 0.41 | 0.20% | -0.02 | 0.90 | -0.06% | -0.25 | 0.20 | -0.35% |

*Results are from a quasi-binomial regression model adjusted for gender, plate, ethnicity and maternal education.

**One methylation outlier was excluded from analyses.

***Sample size is reduced due to missing exposure information for PM$_{2.5}$.
only one of the two loci selected for replication was successful. Prenatal PM$_{10}$ exposure was associated with a 1.3 higher DNA methylation level at cg03579365 in COLEC11 in the analysis using the HM450 platform, whereas the magnitude of the association was smaller using Pyrosequencing and was with 1st trimester PM$_{10}$ exposure instead. Thus while a consistent direction of effect was observed with prenatal PM$_{10}$ exposure, in utero PM$_{10}$ exposure was associated with higher methylation in cg03579365 in the analysis of the HM450 platform, whereas the magnitude of the association was smaller using Pyrosequencing and was with 1st trimester PM$_{10}$ exposure instead. Thus while a consistent direction of effect was observed with prenatal PM$_{10}$ exposure, in utero PM$_{10}$ exposure was associated with higher methylation in cg03579365.
spermatid development [51]. Exposure to titanium dioxide nanoparticles in the testes has been shown to affect sperm formation and to alter TDRD6 expression [52]. In one study, TDRD6 was implicated in atherosclerosis [53]. However, none of these genes have been previously implicated in asthma pathogenesis.

One of the great strengths of this study is the temporal separation of PM exposure assessment, DNA methylation measurement, and childhood health outcomes, enabling the study to truly address a DOHaD hypothesis. Several limitations should also be noted. We chose to focus on the loci in promoter regions, and therefore, we may have missed interesting associations between exposure and methylation in loci outside these regions. We processed HM450 data using methylumi [54] however, use of other processing and data cleaning techniques may produce different results. Although we made every effort to control for potential confounders, we cannot exclude the possibility of residual confounding by some unknown factor that is associated with DNA methylation levels, ambient air pollution, and cardio-respiratory phenotypes. We measured DNA methylation in newborn blood. Although we adjusted for six common cell types using the Houseman method [55], the small changes in methylation observed may still be the result of shifts in cell populations of smaller subtypes [56]. Moreover, the Houseman method is meant for adult blood, and thus cannot address nucleated red blood cells or immature forms present in newborn blood. Only one of two loci evaluated for replication was successful. There may be several reasons for why one of the loci failed to replicate, including the original result was a false positive, differences in the laboratory assay sensitivities and/or measurement error, the fact that we could not adjust for cell fraction in the replication population, and statistical differences between the original and replication analyses leading to differential results. Misclassification of exposure is another limitation since air pollution exposure based on residential address does not capture individual behaviors. Estimation of trimester exposures based on birth certificate reporting of gestational age is prone to error [57]. Despite this inherent measurement error, we observed multiple trimester-specific effects of exposures and future studies specifically designed to capture weekly exposures in pregnancy will help to further narrow the relevant biological window of susceptibility.

Materials and Methods

Study Population

This study was nested in the Children’s Health Study, a longitudinal cohort study of respiratory health [58]. A subset of 737 children was recruited to participate in a sub-study of air pollution and atherosclerosis. Within this sub-study, 273 children had a newborn bloodspot in which DNA methylation was assessed using the Infinium HumanMethylation450 BeadChip (HM450). An additional subset of 280 different children with newborn bloodspots was chosen for a replication population in which DNA methylation was assessed using Pyrosequencing. All subjects had systolic/diastolic blood pressure, supine heart rate, standing height, and weight measured during a classroom visit, and B-mode carotid artery ultrasound was performed. Personal, parental, and socio-demographic characteristics, including maternal smoking during pregnancy, were obtained by parent-completed questionnaire. Children were classified as having asthma if the adult completing the questionnaire reported that a doctor had “ever diagnosed the child as having asthma.” Participants’ parents were asked about previous occurrences of stroke, heart failure, and heart attack or angina. Affirmative responses were coded as having a prior family history of heart disease if an individual responded yes to any of these inquiries. DNA methylation was measured in newborn bloodspots that were obtained from the California Department of Public Health Genetic Disease Screening Program. Birth weight, gestational age, mode of delivery, and other reproductive data were obtained from California birth records. The estimated date of conception was assigned using the birth date and gestational age, corrected for the average 2-week difference between the last menstrual period and conception.

Air Pollution Assessment

The CHS air quality monitoring data [33, 34, 59] and the US EPA Air Quality System (AQS) were used to assign estimates of prenatal air pollution exposures for PM$_{2.5}$ and PM$_{10}$ based on residential address reported on the birth certificate and at the time of the baseline questionnaire. Addresses were geocoded using TeleAtlas Inc.’s Address Point Geocoding Services. Station-specific air quality data were spatially interpolated to each birth residence using inverse-distance-squared weighting. The data from up to four air quality measurement stations were included in each interpolation. Due to the regional nature of PM$_{10}$ and PM$_{2.5}$ concentrations, a maximum interpolation radius of 50 km was used for all pollutants. However, when a residence was located within 5 km of one or more stations with valid observations, the interpolation was based solely on the nearby values. When multiple addresses were reported, the average concentrations were time-weighted to account for portion of year spent at each address. Individuals with incomplete residential histories were excluded from analyses. Prenatal air pollution assignments were successfully made for 241 of the 273 primary participants, with the exception of PM$_{2.5}$, for which we had only 186 subjects with assigned exposure due to lack of monitoring data in some communities. Subjects with and without exposure data were largely similar except for differences in prevalence of in utero smoke exposure and asthma (see online Supplementary material, Table S4). In the replication population, 280 subjects had PM$_{10}$ and 149 had PM$_{2.5}$ measurements available.

The study protocol was approved by the University of Southern California Institutional Review Board and informed, written consent and assent were provided by the parents and children respectively.

Health Measurements

CIMT, heart rate, and blood pressure were assessed by a single physician-imaging specialist from the USC Atherosclerosis Research Unit (ARU) Core Imaging and Reading Center (CIRC). As described previously (Patents 2005, 2006) [60-63], the jugular vein and carotid artery were imaged transversely with the jugular vein stacked above the carotid artery. All images contained internal anatomical landmarks for reproducing probe angulation and a single-lead electrocardiogram was recorded simultaneously with the B-mode image to ensure that CIMT was measured at the R-wave in the cardiac cycle. CIMT was measured along the far (deep) wall of the distal common carotid artery (0.25 cm from the carotid artery bulb) along a standard 1 cm length that was automatically determined by a computer-generated ruler. This method standardizes the timing, location, and distance over which CIMT is measured, ensuring comparability across participants [60-63]. Duplicate scans were
conducted 2.5 days apart on average for CIMT (n = 44) and the intra-class correlation between replicate scans was 0.84.

DNA Methylation

DNA methylation was measured in archived newborn bloodspots as the most proximal biomarker of DNA methylation reflecting the fetal experience. Newborn bloodspots were stored by the state of California at −20°C. Upon receipt, the bloodspots were stored in our lab at −80°C until DNA extraction. Laboratory personnel performing DNA methylation analysis were blinded to study subject information. DNA was extracted from one half of a newborn bloodspot using the QiaAmp DNA blood kit (Qiagen Inc, Valencia, CA) and stored at −80°C. Average yield of DNA from half a spot was 550 ng (ranged from 17 ng to 2342 ng). Generally, 700 ng to 1 μg of genomic DNA from each sample was treated with bisulfite using the EZ-96 DNA Methylation Kit™ (Zymo Research, Irvine, CA, USA), according to the manufacturer’s recommended protocol and eluted in 18 μl. The results of the Infinium HumanMethylation450 BeadChip (HM450) were compiled for each locus as previously described and were reported as beta (β) values [64]. A normal exponential background correction was first applied to the raw intensities at the array level to reduce background noise followed by dye bias correction [54]. We then normalized each sample’s methylation values to have the same quantiles to address sample to sample variability [65].

CpG loci on the HM450 array were removed from analyses if they were on the X and Y chromosomes, or if they contained SNPs, deletions, repeats, or if they have more than 10% missing values, leaving 383,857 probes for analysis. Only probes mapped to the promoter region were included in the analysis yielding 178,500 loci for interrogation and multiple comparison correction. Samples from 273 participants were included for initial analysis of DNA methylation. Thirty-two samples were removed for missing PM2.5 air pollution exposure and an additional one was excluded since many probes for this sample were identified as outliers. This left 240 samples for the primary analysis.

For replication analyses, PCR primers targeting the selected loci were developed using MethPrimer software [66]. Primers were designed to cover the HM450 loci of interest and the specificity of the primer sequences were confirmed using in silico PCR. 250 ng DNA was treated with bisulfite using the EZ-96 DNA Methylation Kit™ as described above and Methylation analyses were performed using the Pyrosequencing (PSQ) HS 96 Pyrosequencing System (Biotage AB, Uppsala, Sweden) as described in previous work [67]. The output from Pyrosequencing is reported as a percent of DNA methylation at each CpG locus. As a quality control check to estimate the bisulfite conversion efficiency, we placed duplicate genomic DNA samples on each bisulfite conversion plate to estimate the internal plate variation of bisulfite conversion and the Pyrosequencing reaction. Conversion efficiency was greater than 95%. We also added universal PCR products amplified from cell line DNA on each Pyrosequencing plate to check the run-to-run and plate-to-plate variation in performing Pyrosequencing reactions. In addition, the Pyrogram peak pattern from every sample was checked to confirm the quality of reaction.

Statistical Analyses

Descriptive analyses were performed to examine the distribution of subject characteristics. Density plots of DNA methylation values from the HM450 BeadArray were created and evaluated for quality control. Outlier DNA methylation values were identified as values that were either greater than the median + 5*SD or less than the median − 5*SD and were removed from analyses.

The association between air pollution exposure and percent DNA methylation was analyzed by a generalized linear regression model using quasi-binomial link family, adjusted for gender, plate, and cell types. Additional sensitivity analyses were conducted to evaluate potential confounding of results by maternal education, ethnicity, and in utero tobacco smoke and were found to be minimal. The following cell types were estimated using the method of Housman et al. [55]: B-lymphocytes, granulocytes, monocytes, natural killer cells, CD4+ T-lymphocytes, and CD8+ T-lymphocytes. The generalized linear regression model was used to address the non-normal distribution of DNA methylation values, which are bounded by 0 and 1 and in many cases heavily skewed toward one end or the other. All regression analyses were adjusted for multiple testing at a false discovery rate (FDR) of 0.15, using the method of Benjamini and Hochberg [68]. A less conservative FDR threshold was chosen because we were employing a two-step screening and replication process and did not want to be too restrictive in the first step.

Finally, we used logistic or linear regression models to evaluate the associations between methylation and childhood health outcomes including asthma, CIMT, and z-score transformed blood pressure (normalized for age, height, and sex [69]), adjusting for maternal education, sex, ethnicity, DNA methylation plate, age at clinic assessment, BMI z-score, and in utero tobacco smoke exposure. These covariates were chosen for adjustment based on a priori knowledge of their associations with health outcomes, with the exception of methylation plate, which was included to adjust for potential batch differences. A similar generalized linear regression model using quasi-binomial link family was used in the replication population to evaluate the association between air pollutants and DNA methylation measured by Pyrosequencing, with adjustment for gender, plate, maternal education, and ethnicity. In utero tobacco smoke was not adjusted because no subjects had exposure. Blood cell fractions could not be estimated for this population because 450K data were not available. All tests assumed a two-sided alternative hypothesis, a 0.15 false discovery rate, and were conducted using the R programming language, version R3.2.2.

Acknowledgements

We would like to express our sincere gratitude to Martin Kharrazi, Steve Graham and Robin Cooley at the California Biobank Program and Genetic Disease Screening Program within the California Department of Public Health for their assistance and advice regarding newborn bloodspots. We are indebted to the school principals, teachers, students and parents in each of the study communities for their cooperation and especially to the members of the health testing field team for their efforts. This work was supported by the following NIH grants: 5K01ES017801, 1R01ES022216, 5P30ES07048, P01ES011627, R01ES014447, R01HL061768, R01HL07680.

Data Availability

Data available upon request.
Competing Financial Interests
Fred Lurmann is employed by Sonoma Technology, Inc., a private company that performed the air pollution exposure assignments.

Supplementary data
Supplementary data is available at EnvEpig online.

References
1. Franchini M, Mannucci PM. Air pollution and cardiovascular disease. Thromb Res 2012;129:230–234.
2. Kelly FJ, Fussell JC. Air pollution and airway disease. Clin Exp Allergy 2011;41:1059–1071.
3. Takizawa H. Impact of air pollution on allergic diseases. Korean J Intern Med 2011;26:262–273.
4. Bianchini E, Giannarelli C, Bruno RM, et al. Functional and structural alterations of large arteries: methodological issues. Curr Pharm Des 2013;19:2390–2400.
5. Chirinos JA. Arterial stiffness: basic concepts and measurement techniques. J Cardiovasc Transl Res 2012;5:243–255.
6. Zhao X, Sun Z, Ruan Y, et al. Personal black carbon exposure influences ambulatory blood pressure: air pollution and cardiometabolic disease (AIRCMD-China) study. Hypertension 2014;63:871–877.
7. Liu C, Fuertes E, Tiesler CM, et al. Prenatal particulate air pollution exposure and cardiovascular disease: the Atherosclerosis Risk in Young Adults (ARYA) study. J Hypertens 2003;16:549–555.
8. Vos LE, Oren A, Uiterwaal C, et al. Adolescent blood pressure and blood pressure tracking into young adulthood are related to subclinical atherosclerosis: the Atherosclerosis Risk in Young Adults (ARYA) study. Am J Hypertens 2011;17:499–505.
9. Patel MM, Miller RL. Air pollution and childhood asthma: recent advances and future directions. Curr Opin Pediatr 2009;21:235–242.
10. Brunst KJ, Ryan PH, Brokamp C, et al. Timing and duration of traffic-related air pollution exposure and the risk for childhood wheeze and asthma. Am J Respir Crit Care Med 2015;192:421–427.
11. Bowatte G, Lodge C, Lowe AJ, et al. The influence of childhood traffic-related air pollution exposure on asthma, allergy and sensitization: a systematic review and a meta-analysis of birth cohort studies. Allergy 2015;70:245–256.
12. Hsu HL, Chiu YM, Coull BA, et al. Prenatal particulate air pollution and asthma onset in urban children: identifying sensitive windows and sex differences. Am J Respir Crit Care Med 2015.
13. Molter A, Simpson A, Berdel D, et al. A multicentre study of air pollution exposure and childhood asthma prevalence: the ESCAPE project. Eur Respir J 2015;45:610–624.
14. Barker DJ. In utero programming of cardiovascular disease. Theriogenology 2000;53:555–574.
15. Gorr MW, Velten M, Nelid TD, et al. Early life exposure to air pollution induces adult cardiac dysfunction. Am J Physiol Heart Circ Physiol 2014;307:H1353–H1360.
16. Weldy CS, Liu Y, Chang YC, et al. In utero and early life exposure to diesel exhaust air pollution increases adult susceptibility to heart failure in mice. Part Fibre Toxicol 2013;10:59.
17. Strickland MJ, Klein M, Correa A, et al. Ambient air pollution and cardiovascular malformations in Atlanta, Georgia, 1986–2003. Am J Epidemiol 2009;169:1004–1014.
18. Damaceno-Rodrigues NR, Veras MM, Negri EM, et al. Effect of pre- and postnatal exposure to urban air pollution on myocardial lipid peroxidation levels in adult mice. Inhal Toxicol 2009;21:1129–1137.
19. Gillman MW, Barker D, Bier D, et al. Meeting report on the 3rd International Congress on Developmental Origins of Health and Disease (DOHaD). Pediatr Res 2007;61:625–629.
20. Inutadap S, Ojeda NB, Dasinger JH, et al. Sex differences in the developmental origins of cardiovascular disease. Physiology (Bethesda) 2014;29:122–132.
21. Kelishadi R, Poursafa P. A review on the genetic, environmental, and lifestyle aspects of the early-life origins of cardiovascular disease. Curr Probl Pediatr Adolesc Health Care 2014;44:54–72.
22. Rodford JL, Torrens C, Siow RC, et al. Endothelial dysfunction and reduced antioxidant protection in an animal model of the developmental origins of cardiovascular disease. J Physiol 2008;586:4709–4720.
23. Stanek LW, Brown JS, Stanek J, et al. Air pollution toxicology—a brief review of the role of the science in shaping the current understanding of air pollution health risks. Toxicol Sci 2011;120 Suppl 1:S8–27.
24. Minelli C, Wei I, Saggo G, et al. Interactive effects of antioxidant genes and air pollution on respiratory function and airway disease: a HuGE review. Am J Epidemiol 2011;173:603–620.
25. Breton C, Marutani A. Air pollution and epigenetics: recent findings. Curr Environ Health Rep 2014;1:35–45.
26. Guo L, Byun HM, Zhong J, et al. Effects of short-term exposure to inhalable particulate matter on DNA methylation of tandem repeats. Environ Mol Mutagen 2014;55:322–335.
27. Kile ML, Fang S, Baccarelli AA, et al. A panel study of occupational exposure to fine particulate matter and changes in DNA methylation over a single workday and years worked in boilermaker welders. Environ Health 2013;12:47.
28. Tarantini L, Bonzini M, Apostoli P, et al. Effects of particulate matter on genomic DNA methylation content and INOS promoter methylation. Environ Health Perspect 2009;117:217–222.
29. Sofer T, Baccarelli A, Cantone L, et al. Exposure to airborne particulate matter is associated with methylation pattern in the asthma pathway. Epigenomics 2013;5:147–154.
30. Breton CV, Salam MT, Wang X, et al. Particulate matter, DNA methylation in nitric oxide synthase, and childhood respiratory disease. Environ Health Perspect 2012;120:1320–1326.
31. Jiang RW, Jones MJ, Sava F, et al. Short-term diesel exhaust inhalation in a controlled human crossover study is associated with changes in DNA methylation of circulating mononuclear cells in asthmatics. Part Fibre Toxicol 2014;11.
32. Guo H, Zhu P, Yan L, et al. The DNA methylation landscape of human early embryos. Nature 2014;511:606–610.
33. Peters JM, Avol E, Gauderman WJ, et al. A study of twelve Southern California communities with differing levels and types of air pollution. II. Effects on pulmonary function. Am J Respir Crit Care Med 1999;159:768–775.
34. Peters JM, Avol E, Navidi W, et al. A study of twelve Southern California communities with differing levels and types of air pollution. I. Prevalence of respiratory morbidity. Am J Respir Crit Care Med 1999;159:760–767.
35. Kohli A, Garcia MA, Miller RL, et al. Secondhand smoke in combination with ambient air pollution exposure is associated with increased CpG methylation and decreased...
expression of IFN-gamma in T effector cells and Foxp3 in T regulatory cells in children. Clin Epigenetics 2012;4:17.

36. Sabourni C, Bollyky J, Nadeau K. Review of environmental impact on the epigenetic regulation of atopic diseases. Curr Allergy Asthma Rep 2015;15:33.

37. Klingbeil EC, Hew KM, Nygaard UC, et al. Polycyclic aromatic hydrocarbons, tobacco smoke, and epigenetic remodeling in asthma. Immunol Res 2014;58:369–373.

38. Mousse IR, Chalbot MC, Pathak R, et al. In vitro toxicity and epigenotoxicity of different types of ambient particulate matter. Toxicol Sci 2015;148:473–487.

39. Janssen BG, Byun HM, Gyselaers W, et al. Placental mitochondrial methylation and exposure to airborne particulate matter in the early life environment: An ENVIRONAGE birth cohort study. Epigenetics 2015;10:536–544.

40. Janssen BG, Godderis L, Pieters N, et al. Placental DNA hypomethylation in association with particulate air pollution in early life. Part Fibre Toxicol 2013;10:22.

41. Boyarri-Olmos R, Hansen S, Henriksen ML, et al. Genetic variation of COLEC10 and COLEC11 and association with serum levels of collectin liver 1 (CL-L1) and collectin kidney 1 (CL-K1). PLoS One 2015;10:e0114883.

42. Selman L, Hansen S. Structure and function of collectin liver 1 (CL-L1) and collectin 11 (CL-11, CL-K1). Immunology 2012;121:851–863.

43. Beltrame MH, Catarino SJ, Goeldner I, et al. The lectin pathway of complement and rheumatic heart disease. Front Pediatr 2014;2:148.

44. Kolarova J, Tangen J, Bens S, et al. Array-based DNA methylation analysis in individuals with developmental delay/intellectual disability and normal molecular karyotype. Eur J Med Genet 2015;58:419–425.

45. Bergeret E, Perrin J, Williams M, et al. TM9SF4 is required for Drosophila cellular immunity via cell adhesion and phagocytosis. J Cell Sci 2008;121:3325–3334.

46. Wang J, Zhang Y, Hou J, et al. Ube2s regulates Sox2 stability and mouse ES cell maintenance. Cell Death Differ 2015;22:4808–4817.

47. Feng R, Wen J. Overview of the roles of Sox2 in stem cell and development. Bio Chem 2015;396:883–891.

48. Gontan C, de Munck A, Vermeij M, et al. Improved common carotid artery echogenic edge tracking for quantifying intima-media thickness from B-mode ultrasound images. Atherosclerosis 2012;217:296–309.

49. Li LC, Dahiya R. MethPrimer: designing primers for methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 2012;13:86.

50. Hodis HN, Mack WJ, LaBree L, et al. Alpha-tocopherol supplementation in healthy individuals reduces low-density lipoprotein oxidation but not atherosclerosis: the Vitamin E Atherosclerosis Prevention Study (VEAPS). Circulation 2002;106:1453–1459.

51. Hodis HN, Mack WJ, Lobo RA, et al. Estrogen in the prevention of atherosclerosis. A randomized, double-blind, placebo-controlled trial. Ann Intern Med 2001;135:939–953.

52. Selzer RH, Hodis HN, Kwong-Fu H, et al. Evaluation of computerized edge tracking for quantifying intima-media thickness of the common carotid artery from B-mode ultrasound images. Atherosclerosis 1994;111:1–11.

53. Selzer RH, Mack WJ, Lee PL, et al. Improved common carotid elasticity and intima-media thickness measurements from computer analysis of sequential ultrasound frames. Atherosclerosis 2001;154:185–193.

54. Triche TJ Jr, Weisenberger DJ, Van Den Berg D, et al. Low-level processing of illumina infinium DNA methylation bead arrays. Nucleic Acids Res 2013;41:e90.

55. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics 2012;13:86.

56. Gao G, Ze Y, Zhao X, et al. Titanium dioxide nanoparticle-induced testicular damage, spermatogenesis suppression, and gene expression alterations in male mice. J Hazard Mater 2013;258:259–133–143.

57. McGeachie M, Ramoni RL, Mychaleckyj JC, et al. Integrative predictive model of coronary artery calcification in atherosclerosis. Circulation 2009;120:2448–2454.