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Epigenetic Marks of Prenatal Air Pollution Exposure Found in Multiple Tissues Relevant for Child Health

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Abstract

Background: Prenatal air pollution exposure has been linked to many adverse health conditions in the offspring. However, little is known about the mechanisms underlying these associations. Epigenetics may be one plausible biologic link. Here, we sought to identify site-specific and global DNA methylation (DNAm) changes, in developmentally relevant tissues, associated with

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Conflict of Interest

Fred Lurmann is employed by Sonoma Technology Inc., Petaluma, CA and has received support from an air quality violations settlement agreement between the South Coast Air Quality Management District, a California state regulatory agency, and BP. The other authors of this paper have no actual or potential competing financial interests

prenatal exposure to nitrogen dioxide (NO_2) and ozone (O_3). Additionally, we assessed whether sex-specific changes in methylation exist and whether DNAm changes are consistently observed across tissues.

Methods: Genome-scale DNAm measurements were obtained using the Infinium HumanMethylation450k platform for 133 placenta and 175 cord blood specimens from Early Autism Risk Longitudinal Investigation (EARLI) neonates. Ambient NO_2 and O_3 exposure levels were based on prenatal address locations of EARLI mothers and the Environmental Protection Agency's AirNOW monitoring network using inverse distance weighting. We computed sample-level aggregate methylation measures for each of 5 types of genomic regions including genomewide, open sea, shelf, shore, and island regions. Linear regression was performed for each genomic region; per-sample aggregate methylation measures were modeled as a function of quantitative exposure level with covariate adjustment. In addition, bumphunting was performed to identify differentially methylated regions (DMRs) associated with prenatal O_3 and NO_2 exposures in each tissue and by sex, with adjustment for technical and biological sources of variation.

Results: We identified global and locus-specific changes in DNA methylation related to prenatal exposure to NO_2 and O_3 in 2 developmentally relevant tissues. Neonates with increased prenatal O_3 exposure had lower aggregate levels of DNAm at CpGs located in open sea and shelf regions of the genome. We identified 6 DMRs associated with prenatal NO_2 exposure, including 3 sex-specific. An additional 3 sex-specific DMRs were associated with prenatal O_3 exposure levels. DMRs initially detected in cord blood samples (n=4) showed consistent exposure-related changes in DNAm in placenta. However, the DMRs initially detected in placenta (n=5) did not show DNAm differences in cord blood and, thus, they appear to be tissue-specific.

Conclusions: We observed global, locus, and sex-specific methylation changes associated with prenatal NO_2 and O_3 exposures. Our findings support DNAm is a biologic target of prenatal air pollutant exposures and highlight epigenetic involvement in sex-specific differential susceptibility to environmental exposure effects in 2 developmentally relevant tissues.

Keywords

epigenetic; DNA methylation; prenatal air pollution exposure; placenta; sex differences; genome-scale

1. Introduction

Air pollution exposure has been consistently associated with increased morbidity and mortality across the life span(Chung et al. 2015; Zigler et al. 2018). When exposed during the prenatal period there is mounting evidence for effects on birth weight and gestational age(Li et al. 2017; Ng et al. 2017), as well as on cognitive development and behavior(Cowell et al. 2015; Fuertes et al. 2016; Jedrychowski et al. 2015). Notably, some of these effects differ by sex. Birth weight related effects of prenatal exposure to particulate matter less than 2.5 microns in diameter (PM2.5) were exacerbated among males, particularly among those with obese mothers(Lakshmanan et al. 2015). Neurodevelopmental consequences of prenatal $PM_{2.5}$ exposure may also differ by sex, with preschool aged females showing reduced memory ability with increasing exposure(Chiu et al. 2016). Male preschool aged subjects, however, had alterations in attention with increasing exposure(Chiu et al. 2016). While the

mechanism underlying these associations is not well understood, research into the biological correlates of air pollution exposure may provide insight into etiologic pathways and avenues for identification of susceptible individuals.

Environmental exposures, like air pollution, may alter cellular states and human health outcomes through epigenetic mechanisms. In adults, global changes in DNA methylation, a type of epigenetic mark, have been observed in blood related to indoor solid fuel exposure(Tao et al. 2014). Candidate gene based studies in elderly men have identified changes in blood methylation levels related to particulate matter exposure (Bind et al. 2014; Bind et al. 2015; Chi et al. 2016; Panni et al. 2016). Investigation of genome-scale, site-specific DNA methylation changes have largely evaluated response to urban pollutants, though examining diesel exhaust exposure among asthmatics(Jiang et al. 2014) or long term exposure to nitrogen oxides (Plusquin et al. 2017). Other studies have explored the effects of prenatal exposure to urban air pollutants on the child epigenome at both the global and site-specific levels. For example, decreased methylation at LINE1 repetitive elements in the child genome were shown to be associated with the following maternal exposures during pregnancy: living closer to a major roadway(Kingsley et al. 2016), exposure to nitrogen dioxide (NO_2)(Breton et al. 2016), and particulate matter with an aerodynamic diameter less than 10μm (PM_{10})(Kingsley et al. 2016).

Site-specific genetic analyses can help identify loci associated with varying levels of air pollutants that have the potential to serve as specific biologic targets for intervention efforts. Therefore, two recent studies took a genome-scale approach to identify specific loci showing differential methylation related to prenatal levels of NO_2 exposure(Gruzieva et al. 2017) or proximity to a major roadway(Kingsley et al. 2016); they identified 10 loci in total. The first study identified 3 loci in mitochondrial genes, related to NO_2 exposure via a meta-analysis of 1,508 cord and peripheral blood samples. The overwhelming majority of samples that contributed to this effort were from European countries(Gruzieva et al. 2017). The later study did not examine specific air pollutants but instead examined roadway distance, a commonly used proxy for traffic exposure (Kingsley et al. 2016). While these studies provide important support for epigenetics as a biologic mechanism relevant to air pollution exposure, only a single exposure was examined in each of the previous publications. While both NO_2 and roadway distance capture urban outdoor exposures, research examining the total gaseous oxidant burden has been limited. Common urban oxidants, NO_2 and ozone (O_3), are chemically linked, though each pollutant has different seasonal, spatial, and diurnal patterns(EPA 2015; Simon et al. 2015; Vijayaraghavan et al. 2014; Xing 2013). In epidemiologic studies, moderate negative correlations between these two pollutants in the US are commonly reported (Council 1991; Kerin et al. 2018; Roberts-Semple D 2012).

Despite previous epidemiology studies showing differences in the health effects of air pollution by sex, DNAm differences by offspring sex, related to air pollutants, have not yet been examined. Finally, the effects of prenatal exposure on a tissue that may filter that exposure from mother to child, the placenta, have not yet been examined. The placenta is vital to fetal growth and development. It also acts as a selective barrier, regulates maternal-fetal exchange, filters and detoxifies chemical exposures, and establishes the overall intrauterine environment during pregnancy. Emerging evidence suggests placental

development and function may influence offspring susceptibility to non-communicable diseases across the life span(Burton et al. 2016; Maccani et al. 2013; Marsit 2016; Paquette et al. 2013). Air pollution exposure during pregnancy has been shown to alter placental structure(Soto et al. 2017), morphology(Veras et al. 2008), vascularization(Dutta et al. 2017; Giovannini et al. 2018; Hettfleisch et al. 2017), and function(Janssen et al. 2015; Kingsley et al. 2017; Saenen et al. 2015; van den Hooven et al. 2012; Wylie et al. 2017).

Animal model and human studies have shown disruption of epigenetics during the developmental period is related to altered placental morphology and physiology(Coan et al. 2008; Huang et al. 2017; Mukhopadhyay et al. 2016; Novakovic et al. 2009; Salas et al. 2004; Sibley et al. 2004; Tunster et al. 2016). However, few studies have examined the relationship between prenatal urban air pollutant exposure and epigenetic changes in placental tissue. Candidate gene studies have shown altered imprinted gene expression related to black carbon and $PM_{2.5}$ (Kingsley et al. 2017), differences in DNAm at the *HSD11B2*(Cai et al. 2017) and *LEP*(Saenen et al. 2017) gene loci related to PM_{10} and $PM_{2.5}$ exposures, respectively. Total placental genomic methylation level differences were associated with particulate air pollution levels during pregnancy(Cai et al. 2017; Janssen et al. 2013; Kingsley et al. 2016). Using a genome-scale approach, locus-specific methylation changes in placenta samples have been associated with residential proximity to a major roadway, a commonly used proxy for air pollution exposure(Kingsley et al. 2016). Given the importance of the placenta as a toxicant filtering tissue and evidence supporting the influence of air pollutants on placenta structure and function, potentially through epigenetic mechanisms, additional genome-scale studies of specific air pollutants are needed.

The purpose of this study was to identify site-specific epigenetic changes, at the genome-scale, related to levels of prenatal exposure to NO_2 and O_3 in both cord blood and placenta. In addition, we sought to assess whether exposure-related changes in DNA methylation differ by offspring sex.

2. Materials and Methods

Our study was conducted among a subset of mother-child pairs (n=192 children) enrolled in *the Early Autism Risk Longitudinal Investigation* (EARLI). EARLI is a prospective study of autism spectrum disorder (ASD) that utilizes an enriched familial risk design, i.e. it enrolls pregnant women who have a previous child with ASD, and thus the new baby sibling is at increased risk for ASD given the high sibling recurrence risk. A detailed description of the EARLI study methods can be found in Newschaffer et al.(Newschaffer et al. 2012). Briefly, EARLI was implemented at four major metropolitan locations across the U.S. (Philadelphia, Baltimore, San Francisco Bay Area, and Sacramento), representing three distinct US regions (Southeast Pennsylvania, Northeast Maryland, and Northern California). Recruitment methods varied by location to capitalize on unique resources at each study site. Enrolled mothers were seen at regular intervals during pregnancy (approximately once a trimester) and at birth to complete interviews that cover a wide range of exposure, medical, and demographic domains, as well as to collect biologic and environmental samples, including cord blood and placenta at birth. The EARLI study sample is racially, ethnically, and socioeconomically diverse(Newschaffer et al. 2012).

2.1. Air Pollution Exposure Measurements

Exposure assignments were based on maternal residences recorded prospectively throughout pregnancy for the EARLI study. When mothers lived at more than one location during this time and the date of relocation was not clear, mothers were re-contacted and asked to clarify dates of the move and the residential location. All residential locations for each subject were standardized and geo-coded using the TeleAtlas US_Geo_2 database and software (Tele Atlas, Inc., Boston, CA, www.geocoded.com). Air quality assignments for O_3 and NO_2 were derived from the US EPA's Air Quality System (AQS) data (www.epa.gov/ttn/airs/airsaqs). The monthly air quality data from monitoring stations located within 50 km of each residence were made available for spatial interpolation of ambient concentrations. The spatial interpolations were based on inverse distance-squared weighting (IDW2) of data from up to four closest stations located within 50 km of each participant residence; however, if one or more stations were located within 5 km of a residence then only data from the stations within 5 km were used for the interpolation. Based on estimates of gestational age from medical record review and dates of reported residence we calculated monthly pregnancy exposures. Some subjects were born before 40 weeks of gestation and thus had a shortened prenatal exposure time line. Monthly exposures were averaged to create the cumulative pregnancy exposure metrics used here.

2.2. Biosample collection and genomic DNA extraction

EARLI study staff were present at each delivery. Umbilical cord blood and placenta biosamples were collected shortly after delivery using standardized protocols, implemented across all sites. Placental biopsy samples from the fetal side of the placenta were collected at each clinical site using Baby Tischler Punch Biopsy Forceps. Sample punches were stored at ambient temperature in RNAlater vials (Qiagen, Cat. No. 76154) and shipped same-day to the Johns Hopkins Biological Repository (JHBR) in Baltimore, Maryland, for storage at −190°C until further processing. Umbilical cord blood samples were collected into EDTA tubes and shipped same-day with a cold pack to JHBR for storage at −80°C. Genomic DNA was extracted from both fetal placenta and cord blood samples at JHBR using a QIAgen QIAsymphony automated workstation with the DSP DNA Midi kit (Cat. No. 937255), as specified by the manufacturer. Genomic DNA was quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific).

2.3. DNA methylation measurements and quality control (QC)

We measured DNA methylation at 485,512 loci using the Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA). For each sample, we bisulfite treated 1 μg of high quality genomic DNA using the Zymo EZ-96 DNA Methylation Kit (Cat. No. D5004), as per the manufacturer's instructions, including the specific modifications for Illumina Infinium HumanMethylation450 BeadChip (450K) processing. A total of 237 samples (133 placenta and 104 cord blood) were sent to the Johns Hopkins Genetic Resources Core Facility (GRCF) for processing on the 450K platform. An additional 59 cord blood samples were processed and hybridized to the 450K at a later date at the Center for Epigenetics at the Johns Hopkins University School of Medicine. Cord blood batches were balanced as best possible on exposure. In addition, we accounted for this

potential source of technical variation in our analyses using surrogate variables (details provided below). Raw .idat files were returned to the study investigators for downstream data preprocessing and quality control (QC) using the minfi package (v. 1.20.2) in R (version 3.3.1). Beta values, ranging from 0 to 1, for 0% to 100% methylated, were computed for each locus(Aryee et al. 2014). Several sample- and probe-level QC measures were applied to each tissue, in parallel. First, samples with overall intensity values less than 11 relative fluorescence units RFU were excluded (n=3 cord blood and n=2 placenta). Next, samples were preprocessed using the Noob method with dye-bias correction(Triche et al. 2013). We removed cross-reactive probes (n = 29,233), probes that measured DNA methylation at known SNP positions and those measuring DNA methylation outside of CpG sites (n = 15,464). Finally, probes with detection P-values > 0.01 in more than 10% of samples were removed from downstream analyses (n = 546 probes in cord; n = 573 probes in placenta samples). Samples that did not have NO_2 or O_3 exposure measurements were also removed from our analytic dataset (n=9 cord blood and n=7 placenta samples). A visual summary of our data cleaning pipeline and the number of samples and probes removed at each step can be found in Figure S1. The final EARLI cord blood analytic dataset used in our analyses contained 440,269 probes and 163 samples. The final EARLI placenta analytic dataset consisted of 440,242 probes and 124 samples. A total of 93 neonates had high quality DNA methylation data for both placenta and cord blood and contributed data to both sets of analyses.

2.4. Empirical estimates of cell heterogeneity

The tissue samples examined in this study are comprised of multiple cell types. Because DNA methylation profiles are cell type specific, cell type-specific DNAm could be related to the outcomes of interest and thus be a source of confounding. Therefore, we used the Bioconductor minfi package and *estimateCellType()* function (Aryee et al. 2014) to empirically estimate the proportion of nucleated red blood cells, B cells, natural killer cells, CD4 positive T cells, CD8 positive T cells, granulocytes, and monocytes, in our cord blood specimens(Bakulski et al. 2016). We then used Pearson correlation to determine the relationship between our empirically derived cord blood cell proportions and prenatal exposure levels. There is no equivalent reference panel or method for placenta tissue, therefore, we used surrogate variable analysis (SVA) to capture potential differences in cell composition; SVA has been shown to accurately reflect differences in cell type proportions across samples and was recently recommended as a robust reference-free method to adjust for differences in mixtures of cell types across samples(McGregor et al. 2016). Our use of SVA more generally to accommodate unmeasured confounding is described further below. (McGregor et al. 2016).

2.5. Surrogate variable analyses (SVA)

Surrogate variable analysis (SVA) was used to remove unwanted technical (e.g. batch effects) and biological sources of variation (e.g. genetic ancestry, cellular composition) from our datasets(Kaushal et al. 2017; Leek and Storey 2007; Leek et al. 2012). More specifically, SVA, version 3.22.0, was performed on the methylation beta values to estimate latent factors influencing DNAm levels. We estimated the number of surrogate variables (SVs) to using the Buja and Eyuboglu (‘be’) algorithm, which identifies how many latent surrogate

variables are present in the data. The SVA estimation models for both cord and placenta air pollution analyses included only the air pollution exposure variable of interest. We checked that estimated SVs were associated with measured potential confounders, e.g. plate and position, to assure that adjustment for SVs in our exposure-DNAm analyses would accommodate measured factors. Because we were also concerned that exposure levels were partially correlated with study site and ethnicity, we also ran additional SV models that included them as measured covariates in the final EWAS model and compared the results to those obtained by SV adjustment alone.

2.6. Global DNA methylation analysis

For each sample, we computed a global measure of methylation by computing a mean beta value, i.e. percent methylation, across all measured loci located in the following genome categories: (a) the genome, (b) open sea regions, (c) shelf regions, (d) shore regions, and (e) island regions. Raw methylation beta values were first adjusted for technical and biological sources of variation using surrogate variable analysis (SVA). Using the adjusted methylation values, we computed mean betas values for each sample across all 450K array probes within each genome category described immediately above. Next, we used the *limma* Bioconductor package (version 3.38.3), to perform linear regression for each global methylation category, each prenatal exposure (NO_2 and O_3), and each tissue type (cord blood and placenta). The per-sample mean adjusted methylation beta values for each global category were modeled as a function of exposure.

2.7. Identification of exposure-related differentially methylated regions (DMRs)

For each tissue and each exposure type, we performed bump hunting(Jaffe et al. 2012) to identify prenatal exposure associated differential methylation. Using beta values, ranging from 0 to 1, we identified regional differences in DNA methylation, within each tissue, related to each prenatal exposure using the *bumphunter* () function of the *minfi* R package (v 1.14.0). There are several advantages to using this region-based approach compared to a single probe approach. First, it leverages known high correlation between methylation levels at neighboring CpG sites(Eckhardt et al. 2006; Irizarry et al. 2008) for efficiency. Second, because genomic windows containing multiple probes are used to identify methylation differences, as opposed to single probes, the method is inherently less susceptible to spurious associations due to measurement error that may arise from single probe technical artifacts. Finally, most functionally relevant changes in DNA methylation have been shown to involve multiple neighboring CpG sites.

For all analyses, the models used to identify differential methylation as a function of continuous measures of prenatal air pollution exposure included the exposure variable of interest (i.e. NO_2 or O_3) and the estimated number of SVs for a specific exposure as covariates to account for unwanted sources of biological and technical variability. For *bumphunter* () we used a cutoff of 0.1 (corresponding to contiguous probes with a minimum 10% directionally consistent change in DNAm) and all other parameters set to their default values. To assess statistical significance and account for multiple testing, we performed 1000 linear bootstrapped permutations via *bumphunter()* and report an FWER value, representing the number of regions in permuted (null) data sets that had an area value as extreme as our

observed exposure-associated DMR(Aryee et al. 2014; Jaffe et al. 2012). Consistent with previous literature(Gerring et al. 2018; Jaffe et al. 2016; Kebir et al. 2017; Ladd-Acosta et al. 2014; Miura et al. 2018), we applied an 0.10 FWER significance threshold to our findings. Similar analyses were performed for males and females, separately, within each tissue to assess potential sex-specific DNAm changes related to exposures. Analyses for Y-chromosome probes (n=52) were assessed among males only. For clarity and interpretability, we report exposure-associated differences in methylation in our main tables and figures by exposure quartile.

2.8. Cross-tissue comparison of significant differentially methylated regions

For the differentially methylated regions (DMRs) identified in our initial genome-scale screens, we performed single-CpG regression analyses in the other tissue examined to assess consistency across cord blood and placenta. We extracted all 450k probes within each significant DMR and compared DNA methylation profiles across cord blood and placental tissue samples using the limma package (Ritchie et al. 2015).

2.9. Examination of CpG loci previously associated with air pollution in other studies

We examined DNA methylation at 3 CpG sites previously reported to be associated with measures of prenatal exposure to NO_2 in cord blood samples from primarily white European neonates(Gruzieva et al. 2017). We evaluated the relationship between DNA methylation at each site and prenatal NO_2 exposure in our EARLI cord blood samples by performing single site regression analyses using the limma package(Ritchie et al. 2015). Similarly, we plotted DNA methylation levels and computed statistical measures for 7 loci shown to be associated with distance from nearest major roadway, a less precise estimate of air pollutants, in placenta tissue(Kingsley et al. 2016). Table S1 provides a detailed description of each of the 10 loci tested.

3. Results

3.1. Study sample characteristics

We observed an inverse correlation between O_3 and NO_2 levels in our analytic dataset and across all study sites (Figure S2). For both cord blood (Table 1) and placenta (Table 2) samples, no significant differences in prenatal exposure to NO_2 or O_3 were found to be related to child sex, gestational age, maternal race, or exposure to maternal smoking during pregnancy. Six of the seven empirically estimated (Bakulski et al. 2016) cord blood cell types including nucleated red blood cells, granulocytes, monocytes, B cells, CD4 positive T cells, and CD8 positive T cells, did not differ significantly by prenatal estimates of air pollutants (Table 1). Cord blood derived estimates of natural killer cells showed a marginally significant association (p=0.01) with levels of prenatal exposure to O_3 but not with NO_2 (P=0.96). We observed significantly higher levels of prenatal exposure to NO_2 among individuals enrolled at the Philadelphia and Baltimore (East Coast) sites compared to those from the California (West Coast) sites for both our placenta and cord blood datasets ($P \leq$ 7.04E-14). Significant differences in O_3 levels by site were also observed ($P \leq$ 3.68E-07). Among the set of 162 infants included in our cord blood analytic dataset, those enrolled at the Kaiser Permanente Northern California site had significantly lower levels of O_3 exposure

compared to those enrolled at the 3 other study sites (Table 1). Similarly, in our placenta analytic dataset, individuals enrolled at the Kaiser site had lower levels of O_3 exposure than those enrolled at the UC-Davis site and had intermediate levels of O_3 exposure relative to both sites located on the East Coast (Table 2). We also observed a marginal association between prenatal levels of NO_2 and maternal ethnicity, with the lowest exposure levels present in mothers reporting Hispanic ethnicity. To account for potential differences in genetic ancestry related to exposure levels, we used SVA and also explicitly adjusted for genetic ancestry in our DNA methylation analyses.

3.2. Impact of prenatal air pollutant exposures on global DNA methylation levels

We identified differences in DNA methylation, at a global scale, related to prenatal O_3 and NO_2 exposures (Table 3 **and** Figure 1). For O_3, we observed a significant decrease in DNA methylation in cord blood at open sea regions (P=0.00162) and in placenta at shelf regions of the genome (P=0.00028). Several other suggestive changes (P≤0.004) in global methylation levels were associated with O_3 exposure including shelf and shore regions in cord blood and shore and island regions in placenta. For NO_2, there were suggestive (P=0.003) decreases in placenta DNA methylation levels for both the genome-wide and island genomic regions associated with increased exposure. No differences in global DNA methylation levels were observed in cord blood related to NO_2 exposure. Scatterplots showing the relationship between region-specific global methylation values and air pollutant exposure levels are provided in Figure S3 and S4.

3.3. Identification of methylation changes related to prenatal air pollutant exposure

SVA analyses estimated 18 and 17 SVs in our cord blood samples for NO_2 and O_3 exposures, respectively (Figure S5), and 20 SVs in our placenta sample for both NO_2 and O_3 and exposures (Figure S6). As shown in Figures S5 and S6, SVs were significantly associated with measured sources of technical and biological variation including batch, plate, array position, race, gestational age, ethnicity, and principal components of ancestry (PCs) derived from genome-wide genotyping arrays. All detected SVs were included as covariates in our downstream statistical analyses to detect methylation differences related to NO_2 and O_3 prenatal exposure levels.

For cord blood, we tested a total of 182,723 genomic regions, representing 440,269 measured loci, and identified one significant DMR (FWER=0.028) associated with increased prenatal exposure to NO_2 (Table 3). As shown in Figure 2A, infants with higher *in utero* exposure to NO_2 showed increased DNA methylation at a *RNF39* intragenic region spanning just over 1Kb relative to infants with lower prenatal NO_2 exposure. The magnitude of DNA methylation difference between individuals with the highest and lowest exposures (quartile 4 (Q4) versus quartile 1 (Q1)) was 3.8%. Because we were concerned that exposure levels were partially correlated with study site and race, we also explicitly adjusted for study site and ancestry in our differential methylation analyses; the regression model coefficients and p-values were consistent with our initial analysis (Figure S7 and Table S2).

In placenta samples, we tested a total of 182,698 genomic regions, representing 440,242 measured loci, and identified two DMRs significantly associated (FWER < 0.05) with levels

of prenatal exposure to NO_2 (Table 3). One DMR, located at the *ZNF442* promoter, showed lower relative methylation levels among individuals with increased prenatal exposure to NO_2 (Figure 3A). DNAm levels at the second DMR, located at the 3' UTR of *PTPRH,* were lower among infants with higher prenatal exposure to NO_2 relative to infants with lower prenatal NO_2 exposure (Figure 3B). No ozone-associated DMRs passed our FWER significance threshold of 0.1. Similar to our approach with cord blood results, explicit adjustment for study site and genetic ancestry gave consistent results with our primary analysis (Figure S8 and Table S3).

3.4. Sex-specific associations between methylation and prenatal air pollutant exposures

To determine whether males and females differ in their epigenetic response to prenatal air pollutant exposure, we performed stratified bump hunting analyses. We first performed SVA to estimate SVs within sex and tissue type samples. We identified 12 SVs in female placenta samples and 13 SVs in male placenta samples for both NO_2 and O_3 exposures. For cord blood, 12 and 11 SVs were estimated for both NO_2 and O_3 exposures among females and males, respectively. As shown in Figures S3 and S4, SVs were associated with known sources of technical and biological variation including batch, plate, array position, race, gestational age, ethnicity, and principal components derived from genome-wide genotyping.

Bump hunting analysis among female cord blood samples (n=81) revealed one suggestive DMR associated with prenatal NO_2 exposure (FWER=0.074) and one DMR significantly associated with prenatal O_3 exposure (FWER=0.004)(Table 3); neither DMR was identified in our male-female combined analysis. More specifically, prenatal exposure to increasing levels of NO_2 was related to decreased DNAm levels in cord blood at the *CYP2E1* locus among females (Figure 2B). Females with the highest levels of exposure, i.e. Q4, had 9.3% less methylation, on average, than females with the lowest levels of exposure, i.e. in Q1. Relative loss of methylation at the *PM20D1* promoter was present among females with increased prenatal exposure to O_3 (Figure 2C). Although not statistically significant, the effects in males were in the opposing direction (i.e., gain of methylation) (Figures 2B and 2C). Bump hunting analysis in female placenta samples (n=54) revealed one DMR (FWER=0.056) at the *SLC25A44* locus associated with lower methylation with increasing levels of prenatal exposure to NO_2, although, the magnitude of change was relatively small at 1.7% (Table 3).

Within male cord blood samples (N=82), we identified one DMR associated with prenatal O_3 exposure levels (FWER=0.027) at the *RNF39* locus (Table 3). As shown in Figure 2D, a 2.6% decrease in methylation, on average, was observed among males with the highest quartile (Q4) of exposure to O_3 but no difference in methylation was observed in females. The same genomic region was associated with NO_2 exposure levels in our combined sex sample where both males and females showed similar DNA methylation patterns related to NO_2 exposure (Figure 2A). In male placenta samples (N=70), we identified two DMRs showing significant differences in DNAm related to exposure to NO_2 or O_3 (Table 3). One DMR was located at the 5' UTR region of the *F11R* gene and showed striking differences in DNAm related to prenatal NO_2 exposure levels; males with in Q4 of prenatal exposure had 12.6% less methylation, on average, than males in Q1 of prenatal NO_2 exposure (Figure 3D

and Table 3). No difference in methylation related to prenatal NO_2 exposure levels was observed at the *F11R* locus in females (Figure 3D). The second DMR we identified in placenta, located in the *STK38* promoter region, showed a 0.7% difference in methylation between individuals with the highest (Q4) and the lowest (Q1) exposures to O_3 (Table 3 and Figure 3E).

3.5. Comparison of exposure-related methylation changes across tissues

In addition to identifying air pollutant related epigenetic changes in specific tissues at birth, we also wanted to determine the extent to which the exposure-related epigenetic changes were consistent across tissues. First, we assessed whether the 4 DMRs related to exposure identified in our cord blood EWAS also showed differences in placenta tissue related to exposure. CpG methylation in all four regions showed a consistent direction of effect across both placenta and cord blood tissues related to prenatal exposure to NO_2 or O_3 (Figure 4) but the magnitude of methylation change was smaller in placenta tissue relative to cord blood. For example, individuals with the highest levels of prenatal exposure to NO_2 (Q4) showed increased DNAm at the *RNF39* locus in both cord blood and placenta tissues relative to individuals with lower exposure (Figure 4A).

We also assessed whether the 5 significant DMRs detected in placenta tissue EWAS showed similar trends in cord blood. For 4 of 5 DMRs, there was no inter-individual variance in DNAm in cord blood (Figure 5A-D) and, thus, no opportunity to show inter-individual differences related to air pollutant exposures. The fifth DMR, at *SLC25A44,* showed similar levels and inter-individual variation across tissues but no clear differences related to prenatal levels of NO_2 exposure (Figure 5E).

3.6. Replication of previous findings in our sample

We specifically examined DNA methylation levels in our EARLI sample at 3 CpG sites previously reported to be associated with prenatal exposure to NO_2 in an independent set of cord blood samples from 3 European and 1 U.S. based sample(Gruzieva et al. 2017) For one of the sites (cg08973675), we observed borderline significance and a consistent direction but smaller magnitude of effect with previous reports; neither of the other 2 CpG sites tested showed significant differences in methylation EARLI samples (Table S1). We also did not observe significant differences in methylation at loci previously associated with major roadway proximity, a proxy for traffic exposure of which NO_2 is a component. We did not observe significant differences in placenta methylation at these sites related to either NO_2 or O_3 levels in EARLI samples (Table S1).

4. Discussion

We identified global and locus-specific changes in DNA methylation related to prenatal exposure to air pollutants NO_2 and O_3 in 2 developmentally relevant tissues. We show, for the first time, that there is a global loss of methylation at open sea and shelf regions among neonates with increased prenatal O_3 exposure. In addition to global methylation changes, we discovered 9 locus-specific DMRs associated with prenatal exposure to NO_2 and O_3. Five of the 9 DMRs were child-sex specific. Comparison of exposure-related differences in

methylation across placenta and cord blood tissues revealed that, as expected, there are tissue-specific and as well as shared DMRs across tissues. Our results provide support for involvement of epigenetic mechanisms in air pollution exposures, which are important across a wide-range of adverse health outcomes such as asthma, neurodevelopmental disorders, and metabolic or inflammatory conditions. The regions we identified can be examined in futures studies focused on investigating epigenetic mechanisms in air pollution-disease associations. Finally, our findings can also inform development of prenatal air pollutant exposure biomarkers that could help identify infants at high-risk of adverse health outcomes or could be used in epidemiology studies that are unable to collect more traditional measures of prenatal exposure.

The 4 cord blood DMRs are located in 3 genes: *RNF39, CYP2E1, PM20D1.* One DMR, at the *RNF39* gene locus, was significantly associated with both NO_2 and O_3 levels but showed opposite directions of effect. These findings are consistent given inverse correlations commonly observed between NO_2 and O_3(Council 1991; Kerin et al. 2018; Roberts-Semple D 2012). The 5 placenta DMRs are located in 5 genes: *ZNF442, PTPRH, SLC25A44, F11R,* and *STK38.* Several of these have been shown to be involved in immune and inflammatory processes; these processes have been implicated as biologic targets of air pollutant exposure in independent studies(Johannesson et al. 2014; Ruckerl et al. 2014). For example, *RNF39* is located within the Major Histocompatibility Complex (MHC) class I region and methylation changes in this region have been associated with poor vaccination response to Hepatitis B virus(Lu et al. 2014), multiple sclerosis(Maltby et al. 2017), and systemic lupus erythematosus (SLE) with malar rash(Renauer et al. 2015). *F11R* is part of the inflammatory response biologic pathway, is integral to hematopoietic stem cell fate(Kobayashi et al. 2014), and has been associated with rheumatoid arthritis(Fang et al. 2016). *PTPRH* has been linked to colitis and inflammation in the gastrointestinal tract Other genes we identified are critical regulators of mitochondrial function, energy production and use, and metabolism(Gao et al. 2017; Lee 2016; Lin et al. 2018; Long et al. 2016; Mok et al. 2018; Padilla et al. 2014). Although the specific NO_2-associated genomic regions and genes we identified differ from those reported in Gruzieva et al, they consistently identify changes in genes related to mitochondrial function (Gruzieva et al. 2017). Finally, *CYP2E1,* identified here in cord blood, is a member of the cytochrome P450 enzyme family that metabolizes endogenous substrates including benzene, carbon tetrachloride, ethylene glycol, and nitrosamines.

Our study also examined whether effects of prenatal air pollutant exposure on DNA methylation differ by offspring sex. We observed female-specific differences in cord blood methylation at the *CYP2E1* locus related to NO_2 only. This is interesting in light of observed sexual dimorphic patterns of methylation at this gene locus in mice(Penaloza et al. 2014). Although not statistically significant, methylation plots at this locus revealed the opposite direction of effect among male offspring. This may, at least in part, explain why this gene was not identified in previous studies of methylation changes related to NO_2 exposure when males and females were combined. In cord blood, we also observed male-specific differences in methylation related to O_3 exposure at the *RNF39* gene locus and female-specific differences at the *PM20D1* gene locus among females only. Similarly, in placenta, we identified a striking change in methylation, at the *F11R* gene locus, related to NO_2 exposure among male offspring only. This provides provocative support for a potential

differential response of males and females to prenatal NO_2 and O_3 exposure. Future work is needed to explore potential hormone interactions and whether these DNA methylation changes are related to later differences in disease prevalence rates among males and females across the life course.

The magnitude of change in methylation between exposure quartiles 1 and 4 was greater than 5% for most of the DMRs we identified. However, there were a few DMRs with a relatively small magnitude of change in methylation. Emerging evidence from the environmental epidemiology field suggests that small magnitudes of change in DNA methylation can be highly reproducible and biologically relevant (Breton et al. 2017; Giri and Hollinger 1979; Joubert et al. 2016; Ramakrishnan et al. 1981).

A unique aspect of our study was the ability to examine exposure-related methylation changes across 2 developmentally relevant tissues – cord blood and placenta – among the same study population. Given the role of epigenetics in cell differentiation and function these tissues clearly will have different patterns of methylation, overall. Our main question was whether placenta and cord blood tissue matrices are uniquely or similarly affected by prenatal air pollutant exposure during development. Understanding the similarities and differences can inform our understanding of epigenetic mechanisms of exposure on health as well as the utility of cord blood as a surrogate tissue for exposure-related methylation changes when placenta is not available, i.e. as a biomarker. To set our expectations regarding the extent to which we would see similar epigenetic changes across these 2 tissue matrices, we consider their function and physiology. The cord blood examined in this study was obtained from the umbilical vein and carries nutrients, oxygen, and unfiltered toxicants from the placenta to the fetus. As such, we would expect that exposure-related changes identified in cord blood may also be present in placenta tissue. Although not statistically significant, all 4 of the DMRs we identified in cord blood showed a consistent direction and attenuated magnitude of effect in placenta tissue. The attenuated methylation effect sizes could have decreased our ability to reach statistical significance in our placenta analyses. These observations suggest that exposure-related epigenetic differences observed in cord blood may also be present in placenta tissue, consistent with our expectations. This also suggests that cord blood may serve as a proxy tissue for methylation changes at certain genomic regions and exposures. Placenta-specific changes in DNA methylation are also expected due to its unique role in toxicant detoxification and metabolism that prevents their transfer to the fetal compartment. The air pollutant associated DMRs discovered in our placenta tissue analyses appear to be tissue-specific. We observed no inter-individual differences in methylation and no exposure-related differences in methylation. Thus, our cross-tissue observations are consistent with *a priori* expectations based on placenta and cord blood tissue functions and physiology.

Our EARLI samples did not replicate findings from previous studies. Despite the lack of locus-specific replication, both our study and Gruzieva et al identified NO_2-related methylation changes in genes involved in mitochondrial function(Gruzieva et al. 2017). There are several explanations for the lack of locus-specific replication. It is possible that exposure sources and/or absolute exposure levels differed across the studies. The overwhelming majority (85%) of the samples that contributed data for the previous meta-

analysis of cord blood and neonate blood were from European countries(Gruzieva et al. 2017). In fact, the 2 top loci reported were only assessed in European cohorts. In our sample, we also did not observe consistent differences in DNA methylation at sites previously reported to be associated with proximity to major roadway. Although often used as a proxy for air pollution exposure, it is a very different exposure than what was examined here (NO_2) which could explain the differences in our findings.

We adjusted for cell heterogeneity using surrogate variable analysis (SVA). In placenta, this was necessary to accommodate cell type heterogeneity where no current cell type specific reference panel exists for deconvolution. We used SVA in blood and placenta samples to further accommodate potential confounding from measured and unmeasured technical and biological factors(Teschendorff and Zheng 2017). Cell type heterogeneity is most often considered as a source of potential type I error but failure to adjust for cell heterogeneity has been shown to also result in type II errors(Teschendorff et al. 2017; Teschendorff and Zheng 2017; Zheng et al. 2017). Although unlikely, it is possible that some differences in cord or placenta cell composition were not captured via SVA. Methylation levels at the *PM20D1* gene locus identified in this study have specifically been evaluated elsewhere and shown not to be associated with blood cell heterogeneity(Adalsteinsson et al. 2012). Future studies examining methylation in single cell types, for each tissue type, could be undertaken to further elucidate whether specific cells are important in regulating epigenetic mechanisms of prenatal air pollutant exposure. Even if cell composition shifts underlie air pollution-methylation associations, it is important to understand the biologic changes influenced by prenatal air pollutant exposure in cord blood and placenta tissues.

There are several methodological limitations in measurement to note. While the 450K is a reliable, reproducible, and cost-effective platform to measure genome-scale DNA methylation, it does not measure methylation at every CpG site present in the genome. Future studies with comprehensive genome-wide methylation measurements are needed to fully elucidate the complete set of genomic regions showing methylation changes associated with prenatal air pollutant exposure. Also, while our air pollution exposure methods rely on standard practices for exposure assessment, we did not conduct personal air exposure monitoring or collect time – activity data as part of this study. As such, our assignments are all based on home residence and may not account for variation in behavior patterns and exposures outside the home. Examination of specific windows and/or temporal changes in air pollutant exposure during pregnancy can be incorporated into future analyses and new methods that enable methylation analyses to appropriately model exposure correlations over time will advance this area of research.

4.1. Conclusions

We identified differences in DNA methylation in cord blood and placenta, associated with prenatal exposure to air pollutants O_3 and NO_2. Additional regions with differential methylation by prenatal exposure were child sex-specific. The genes we identified suggest that altered mitochondrial, inflammation, and metabolism biology are linked to prenatal air pollutant exposure and, furthermore, that these effects and relationships may vary by tissue of interest and sex. Our findings contribute to the growing literature that suggests DNA

methylation is a biologic target of prenatal exposure to NO_2 and O_3 air pollutants and define specific molecular effects of air pollution that may be important mediators of adverse health outcomes and differences and also useful birth biomarkers of prenatal exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ASD	autism spectrum disorder
DNAm	DNA methylation
DMR	differentially methylated region
EARLI	Early Autism Risk Longitudinal Investigation
NO_2	nitrogen dioxide
O_3	ozone
SVA	surrogate variable analysis

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Highlights

3 to 5 bullet points (maximum 8_5 characters, including spaces, per bullet point).

- Prenatal air pollutant exposure is associated with neonate DNA methylation changes
- Increased prenatal ozone exposure associates with global losses of methylation
- Locus-specific air pollutant methylation changes occur in placenta and cord blood
- Some locus-specific methylation changes are sex-specific

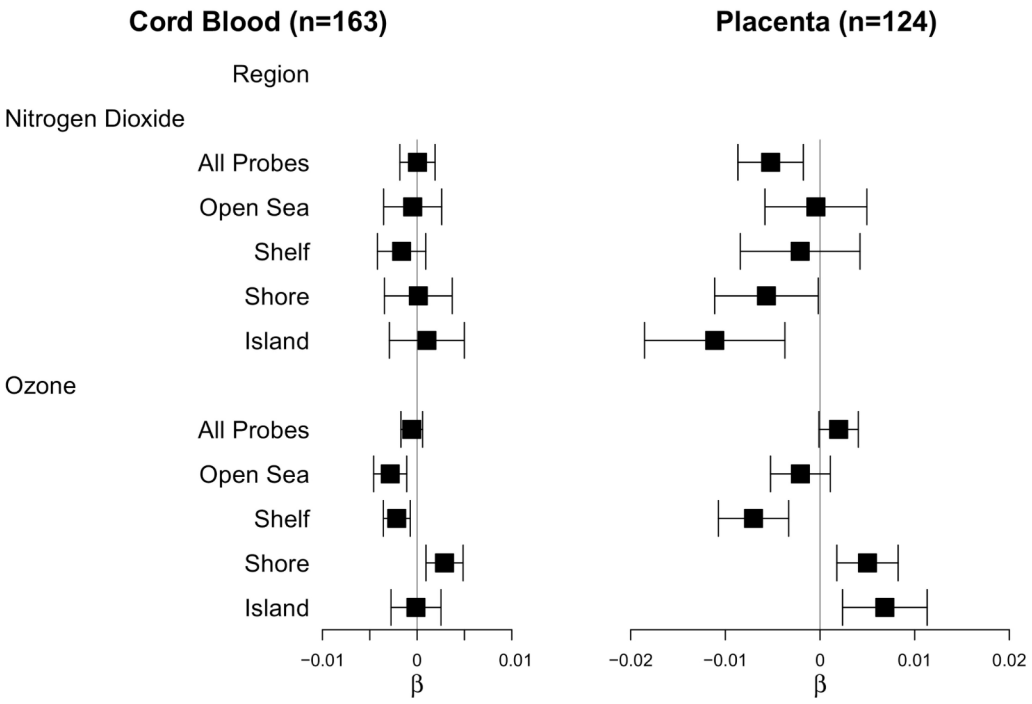
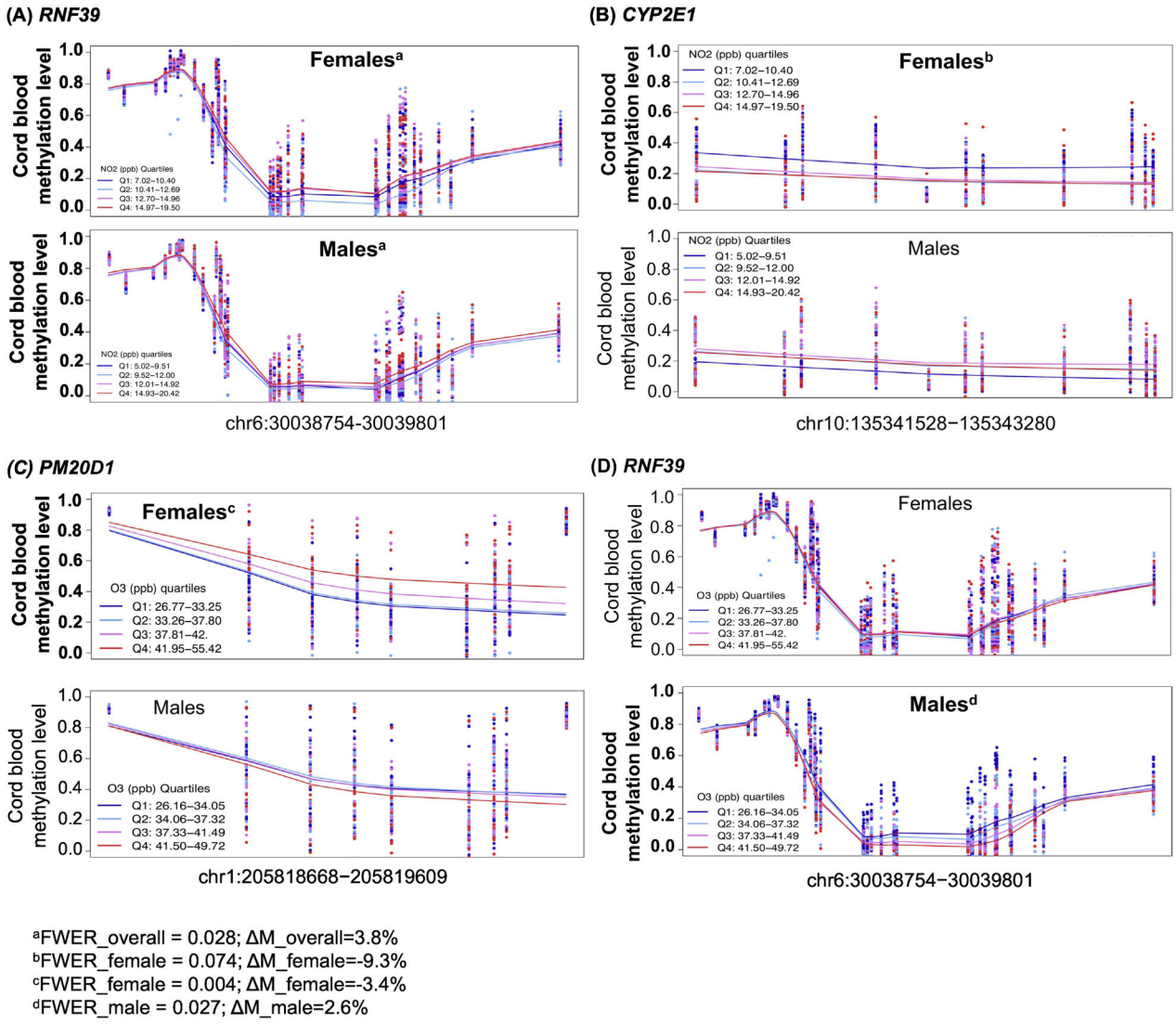


Figure 1.

Forest plot showing differences in global methylation levels in cord blood (left) and placenta (right) related to prenatal air pollutant exposure by type of genomic region. For each exposure and tissue type, boxes represent the estimated linear regression coefficient, i.e. the change in mean DNA methylation for each part per billion (ppb) increment of exposure. Horizontal lines denote the 95% confidence intervals for each regression estimate.



[a]FWER_overall = 0.028; ΔM_overall=3.8%
[b]FWER_female = 0.074; ΔM_female=-9.3%
[c]FWER_female = 0.004; ΔM_female=-3.4%
[d]FWER_male = 0.027; ΔM_male=2.6%

Figure 2.

Cord blood differentially methylated regions (DMRs) associated with prenatal exposure to air pollutants. *(A)* Relative hypermethylation at the *RNF39* gene locus in cord blood is associated with increasing levels of prenatal exposure to nitrogen dioxide (NO_2) in both males and females. *(B)* Increasing prenatal exposure to NO_2 is associated with decreased levels of DNAm at the *CYP2E1* locus in females. *(C)* Decreased prenatal exposure to ozone (O_3) is associated with less DNAm at the *PM20D1* locus among females. *(D)* Increased prenatal exposure to ozone (O_3) is associated with decreased DNAm at the *RNF39* locus among males. For each plot, genomic position is provided on the x-axis and methylation level is plotted on the y-axis, with 0 denoting 0% methylated and 1 denoting 100% methylated. Each point represents the methylation level at a single CpG site for one sample. Lines represent the loess smoothed methylation mean values across the region. Red denotes individuals in quartile 4, i.e. they have the highest levels of prenatal NO_2 exposure. Blue represents individuals in quartile 1, with the lowest levels of prenatal NO_2 exposure. Light blue and pink colors denote individuals in quartiles 2 and 3, respectively, with intermediate

levels of exposure. Bold font denotes the analytic sample where the significant difference in methylation was detected. Family wise error rate (FWER) and magnitude of methylation change (ΔM), between the highest and lowest quartiles of exposure, are provided at the bottom of the figure.

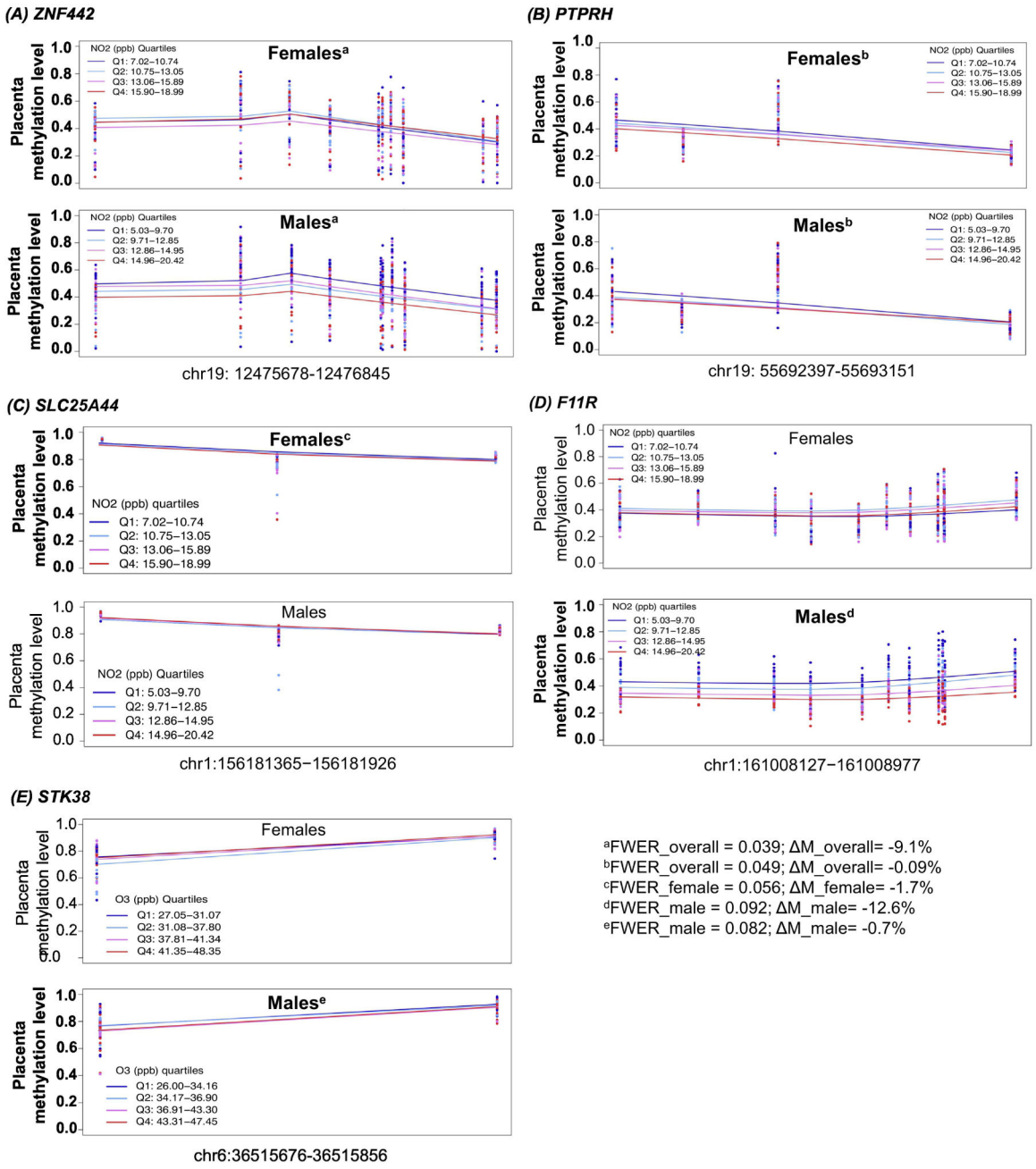


Figure 3.

Placenta differentially methylated regions (DMRs) associated with prenatal exposure to air pollutants. Relative hypomethylation at the (A) *ZNF442* and (B) *PTPRH* gene loci in placenta tissue is associated with increasing levels of prenatal exposure to nitrogen dioxide (NO_2), detected in our combined sample. *(C)* Relative loss of methylation at the *SLC25A44* gene locus is associated with increasing prenatal NO_2 exposure levels, detected via female stratified analyses. Relative hypomethylation at the (D) *F11R* and (E) *STK38* gene loci is associated with increasing prenatal exposure to NO_2 and O_3, respectively, among males. For each plot, genomic position is provided on the x-axis and methylation level is plotted on the

y-axis, with 0 denoting 0% methylated and 1 denoting 100% methylated. Each point represents the methylation level at a single CpG site for one sample. Lines represent the loess smoothed methylation mean values across the region. Red denotes individuals in quartile 4, i.e. they have the highest levels of prenatal exposure. Blue represents individuals in quartile 1, with the lowest levels of prenatal exposure. Light blue and pink colors denote individuals in quartiles 2 and 3, respectively, with intermediate levels of exposure. Bold font denotes the analytic sample where the significant difference in methylation was detected. Family wise error rate (FWER) and magnitude of methylation change (ΔM), between the highest and lowest quartiles of exposure, are provided at the bottom of the figure.

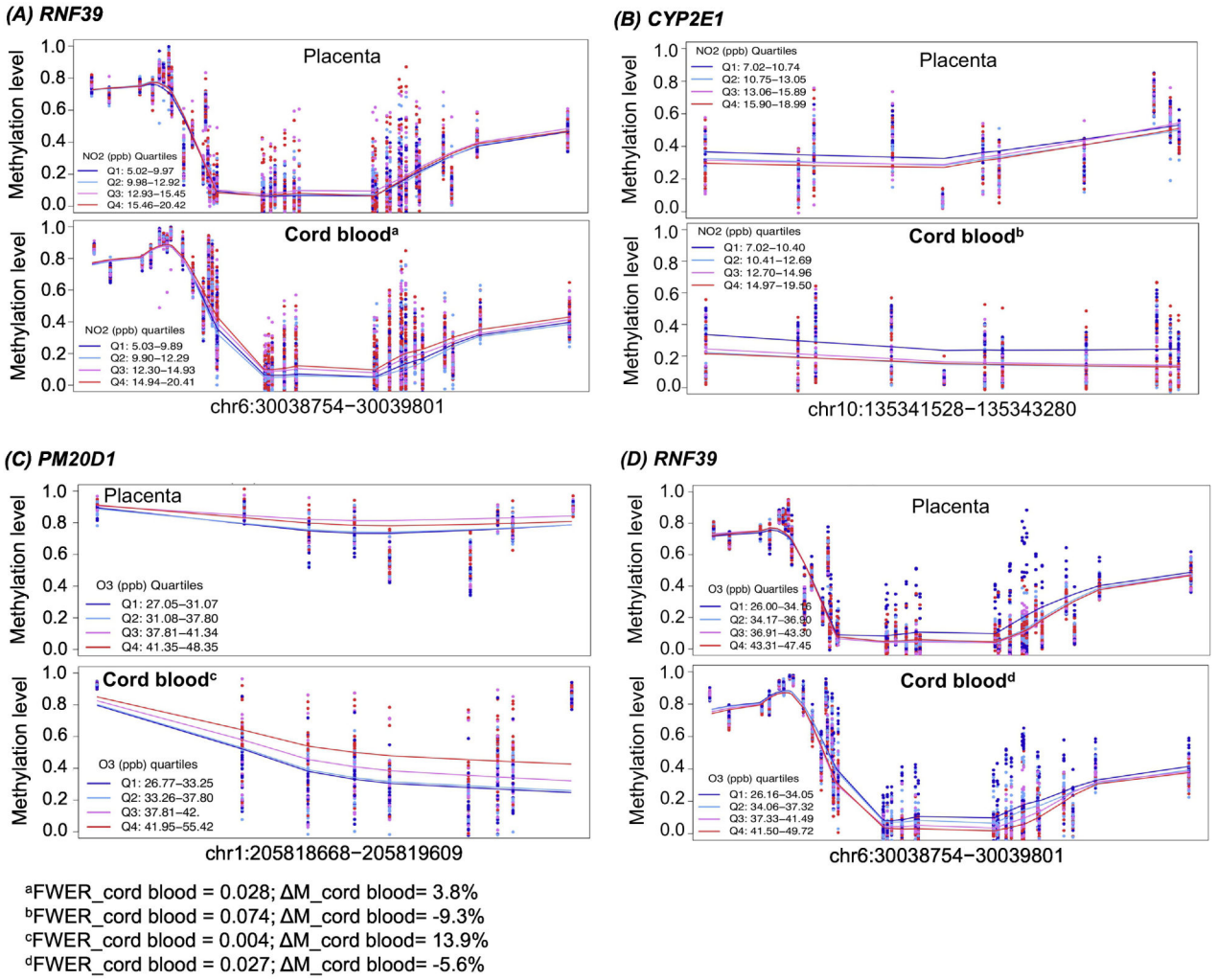


Figure 4.

Air pollutant associated DMRs, detected in cord blood samples, show similar exposure-related patterns of DNA methylation in placenta tissue. Methylation plots for DMRs associated with varying levels of prenatal nitrogen dioxide (NO_2) exposure at the (A) *RNF39* and (B) *CYP2E1* gene loci. Methylation plots at the (C) *PM20D1* and (D) *RNF39* gene regions, associated with prenatal exposure to ozone (O_3). For each plot, genomic position is provided on the x-axis and methylation level is plotted on the y-axis, with 0 denoting 0% methylated and 1 denoting 100% methylated. Each point represents the methylation level at a single CpG site for one sample. Lines represent the loess smoothed methylation mean values across the region. Red denotes individuals in quartile 4, i.e. they have the highest levels of prenatal exposure. Blue represents individuals in quartile 1, with the lowest levels of prenatal exposure. Light blue and pink colors denote individuals in quartiles 2 and 3, respectively, with intermediate levels of exposure. Family wise error rate (FWER) and magnitude of methylation change (ΔM), between the highest and lowest quartiles of exposure, are provided at the bottom of the figure.

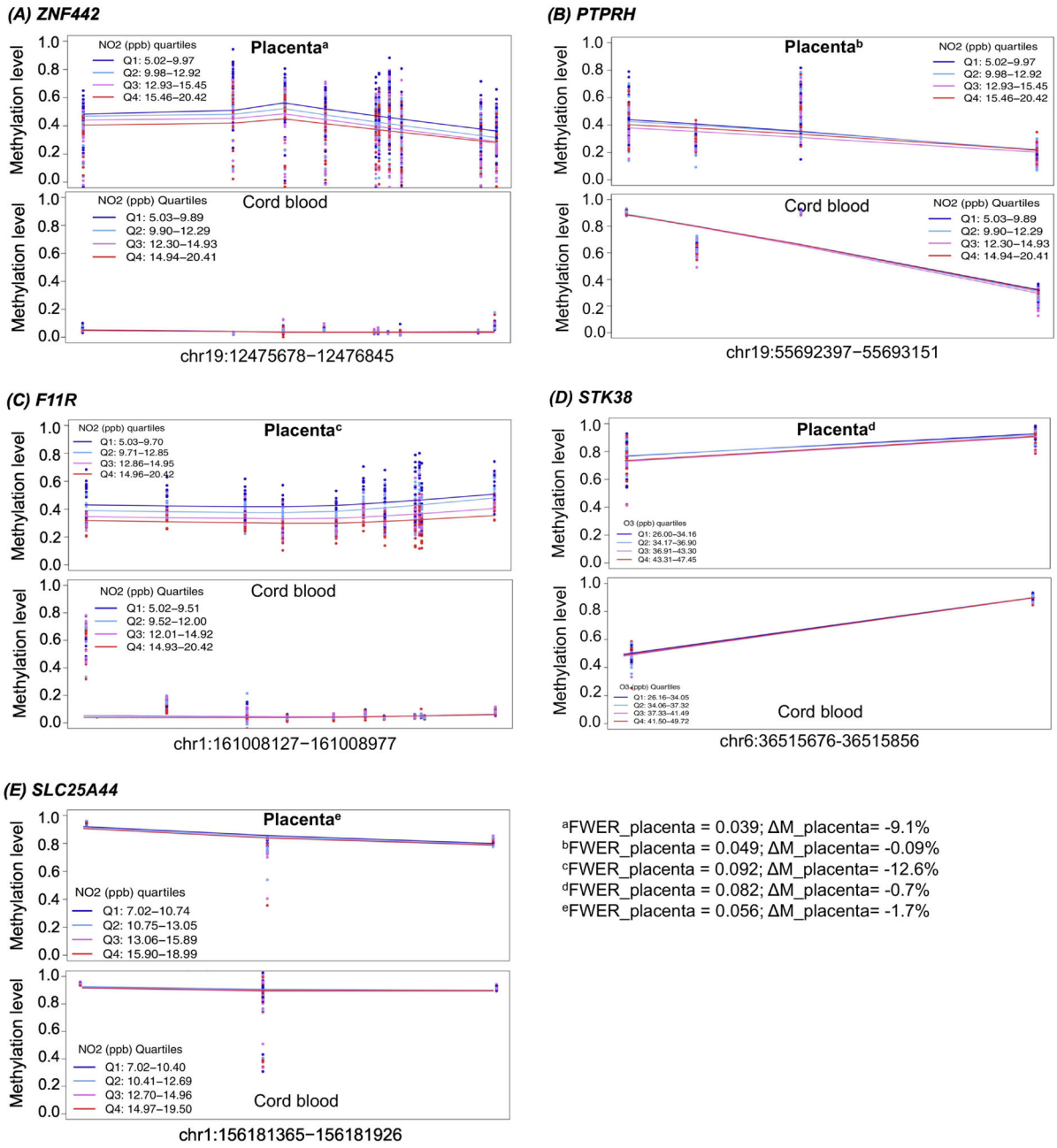


Figure 5.

Air pollutant associated DMRs, detected in placenta tissue, show little inter-individual variation in methylation and no exposure-related patterns of DNA methylation in cord blood samples. Methylation plots for the (A) *ZNF442*, (B) *PTPRF4,* (C) *F11R*, (D) *STK38,* and (E) *SLC25A44* genomic regions. For each plot, genomic position is provided on the x-axis and methylation level is plotted on the y-axis, with 0 denoting 0% methylated and 1 denoting 100% methylated. Each point represents the methylation level at a single CpG site for one sample. Lines represent the loess smoothed methylation mean values across the region. Red denotes individuals in quartile 4, i.e. they have the highest levels of prenatal exposure. Blue represents individuals in quartile 1, with the lowest levels of prenatal

exposure. Light blue and pink colors denote individuals in quartiles 2 and 3, respectively, with intermediate levels of exposure. Family wise error rate (FWER) and magnitude of methylation change (ΔM), between the highest and lowest quartiles of exposure, are provided at the bottom of the figure.

Table 1.

Descriptive characteristics of Early Autism Risk Longitudinal Investigation (EARLI) cord blood DNA methylation samples, by prenatal air pollutant exposure levels

		Nitrogen dioxide (NO_2)[a]		Ozone (O_3)[b]	
	n (%)	**Mean ± SD**	***P***	**Mean ± SD**	***P***
Total	163	12.27 ± 3.18		37.72 ± 5.77	
Child sex					
Male	82 (50.3)	12 ± 3.4	0.357	37.8 ± 5.2	0.837
Female	81 (49.7)	12.5 ± 3		37.6 ± 6.3	
Maternal smoking[c]					
No	160 (98.2)	12.2 ± 3.2	0.493	37.7 ± 5.8	0.819
Yes	2 (1.8)	13.9 ± 3.5		38.2 ± 3.3	
Maternal Race[d]					
White	89 (54.6)	12.65 ± 3.24	0.023	38.74 ± 5.70	0.025
Black	14(8.6)	13.97 ± 2.92		38.51 ± 5.23	
Asian	14 (8.6)	11.79 ± 2.44		33.99 ± 3.97	
Multiple	31 (19)	11.10 ± 3.29		37.19 ± 5.81	
Other or Missing	15 (9.2)	11.23 ± 2.59		35.47 ± 6.36	
Maternal Ethnicity[d]					
Hispanic or Latino	38 (23.3)	10.95 ± 3.13	0.0063	36.02 ± 6.62	0.089
Not Hispanic or Latino	110 (67.5)	12.74 ± 3.10		38.42 ± 5.28	
Other	6 (3.7)	13.53 ± 2.54		37.86 ± 7.95	
Missing	9 (5.5)	11.18 ± 3.35		36.25 ± 5.29	
Cell composition[e] (% Mean ± SD)					
B cell		11.3 ± 3.8	0.898	11.3 ± 3.8	0.297
CD4+T cell		19.8 ± 8.3	0.667	19.8 ± 8.3	0.38
CD8+T cell		13.2 ± 4.2	0.126	13.2 ± 4.2	0.0809
Granulocyte		42.3 ± 12.6	0.683	42.3 ± 12.6	0.189
Monocyte		8.4 ± 2.5	0.4	8.4 ± 2.5	0.413
Natural killer cell		0.5 ± 1.2	0.957	0.5 ± 1.2	0.0133
Nucleated red blood cell		10.1 ± 5.4	0.885	10.1 ± 5.4	0.0673
EARLI study site					
Drexel	36 (22.1)	14.8 ± 2.9	4.91E-18	39.2 ± 5.2	1.69E-07
Johns Hopkins	39 (23.9)	14.1 ± 2.1		39.9 ± 4	
Kaiser Permanente	49 (30.1)	10.7 ± 2.7		33.9 ± 5.2	
UC-Davis	39 (23.9)	10.1 ± 2.1		39 ± 6.3	
Gestational age (min, max)	39.4 (39,40.2)		0.382		0.449

Note: SD, standard deviation; P, p-value

[a] 24-hour average NO_2 concentration (parts per billion) during pregnancy

[b] Average daily 8-hour maximum O_3 concentration (parts per billion) during pregnancy

[c] Sustained active maternal smoking during pregnancy, defined as having smoked cigarettes for more than 4 months during pregnancy

[d] Derived from maternal self-report data collected during pregnancy

[e] Empirically estimated using the Bakulski et al. method(Bakulski et al. 2016)

Table 2.

Descriptive characteristics of Early Autism Risk Longitudinal Investigation (EARLI) children with placenta sample DNA methylation data, by prenatal air pollutant exposure levels

		Nitrogen dioxide (NO_2)[a]		Ozone (O_3)[b]	
Characteristic	**n (%)**	**Mean ± SD**	**P**	**Mean ± SD**	**P**
Total	124	12.58 ± 3.41		37.42 ± 5.72	
Sex					
Male	70 (56.5)	12.3 ± 3.5	0.285	37.7 ± 5.5	0.519
Female	54 (43.5)	13 ± 3.2		37 ± 6	
Maternal smoking[c]					
No	121 (97.6)	12.5 ± 3.4	0.57	37.4 ± 5.8	0.715
Yes	3 (2.4)	13.9 ± 3.5		38.2 ± 3.3	
Maternal Race[d]					
White	61 (49.2)	12.89 ± 3.31	0.0257	38.92 ± 5.28	0.0182
Black	10 (8.1)	15.07 ± 2.73		37.38 ± 5.21	
Asian	10 (8.1)	11.16 ± 3.19		33.07 ± 4.22	
Multiple	21 (16.9)	11.58 ± 3.75		37.88 ± 5.91	
Other or missing	22 (17.7)	12.19 ± 3.25		34.81 ± 6.03	
Maternal Ethnicity[d]					
Hispanic or Latino	30 (24.2)	11.06 ± 3.15	0.022	36.69 ± 6.41	0.389
Not Hispanic or Latino	77 (62.1)	12.86 ± 3.36		38.12 ± 5.30	
Other	4 (3.2)	14.41 ± 2.71		35.63 ± 7.00	
Missing	13 (10.5)	13.9 ± 3.56		35.46 ± 5.97	
EARLI study site					
Drexel	29 (23.4)	15.2 ± 2.6	7.04E-14	39 ± 4.2	3.68E−07
Johns Hopkins	30 (24.2)	14.5 ± 1.8		40.6 ± 3.9	
Kaiser Permanente	37 (29.8)	10.7 ± 3.3		33.5 ± 5.1	
UC-Davis	28 (22.6)	10.2 ± 2.4		37.6 ± 6.7	
Gestational age (min, max)	39.4 (39, 40)		0.609		0.161

Note: SD, standard deviation; P, p-value

[a] 24-hour average NO_2 concentration (parts per billion) during pregnancy

[b] Average daily 8-hour maximum O_3 concentration (parts per billion) during pregnancy

[c] Sustained active maternal smoking during pregnancy, defined as having smoked cigarettes for more than 4 months during pregnancy

[d] Derived from maternal self-report data collected during pregnancy

Table 3.

Global DNA methylation levels are significantly associated with differential prenatal exposure to nitrogen dioxide (NO_2) and ozone (O_3).

Tissue, exposure, genomic region	No. probes[a]	ΔM[b]	CI.U	CI.L	P-value[c]
Cord blood (n=163)					
Nitrogen dioxide (NO_2)					
All probes	429,809	0.00004	0.00190	−0.00182	0.96767
Open sea	151,976	−0.00047	0.00260	−0.00354	0.76316
Shelf	40,207	−0.00164	0.00091	−0.00420	0.20648
Shore	100,827	0.00013	0.00371	−0.00345	0.94425
Island	136,799	0.00103	0.00499	−0.00293	0.60848
Ozone (O_3)					
All probes	429,809	−0.00057	0.00058	−0.00171	0.33195
Open sea	151,976	−0.00284	−0.00109	−0.00460	**0.00162**
Shelf	40,207	−0.00215	−0.00073	−0.00357	*0.00318*
Shore	100,827	0.00290	0.00486	0.00094	*0.00403*
Island	136,799	−0.00012	0.00252	−0.00276	0.92797
Placenta (n=124)					
Nitrogen dioxide (NO_2)					
All probes	429,782	−0.00522	−0.00176	−0.00867	*0.00342*
Open sea	151,963	−0.00043	0.00495	−0.00581	0.87422
Shelf	40,199	−0.00201	0.00422	−0.00842	0.51267
Shore	100,825	−0.00566	−0.00019	−0.01113	0.04284
Island	136,795	−0.01112	−0.00371	−0.01853	*0.00359*
Ozone (O_3)					
All probes	429,782	0.00197	0.00405	−0.00011	0.06318
Open sea	151,963	−0.00207	0.00108	−0.00523	0.19637
Shelf	40,199	−0.00702	−0.00331	−0.01074	**0.00028**
Shore	100,825	0.00502	0.00825	0.00178	*0.00266*
Island	136,795	0.00685	0.01132	0.00238	*0.00295*

Notes: CI.U, upper bound of confidence interval; CI.L, lower bound of confidence interval

[a] Total number of 450K probes located in the specified genomic region that contributed to the global measure of DNA methylation

[b] Difference in mean DNA methylation for each part per billion (ppb) increment of exposure. Positive and negative values represent an increase or decrease in mean methylation per unit of exposure, respectively.

[c] P-values that pass a Bonferroni corrected p-value threshold (p<0.0025) are bolded and suggestive (p<0.005) differences are italicized

Table 4.

Significant differentially methylated regions (DMRs) associated with prenatal exposure to nitrogen dioxide (NO_2) and ozone (O_3) levels, identified in cord blood

					Gene annotation		
Tissue type	**Exposure**	**Genomic location**	**ΔM[a]**	**FWER[b]**	**Symbol**	**Distance[c]**	**Location**
Cord blood							
	Nitrogen dioxide (NO_2)						
	All (n=163)	chr6:30038754-30039801	3.8%	0.028	*RNF39*	3827	intragenic
	Female (n=81)	chr10:135341528-135343280	−9.3%	0.074	*CYP2E1*	661	5’ UTR
	Male (n=82)	*no significant DMRs identified*					
	Ozone (O_3)						
	All (n=163)	*no significant DMRs identified*					
	Female (n=81)	chr1:205818668-205819609	13.9%	0.004	*PM20D1*	0	promoter
	Male (n=82)	chr6:30038754-30039801	−5.6%	0.027	*RNF39*	3827	intragenic
Placenta							
	Nitrogen dioxide (NO_2)						
	All (n=124)	chr19: 12475678-12476845	−9.1%	0.039	*ZNF442*	0	5’ UTR
		chr19: 55692397-55693151	0.9%	0.049	*PTPRH*	27723	3’ UTR
	Female (n=54)	chr1: 156181365-156181926	−1.7%	0.056	*SLC25A44*	17635	Exon
	Male (n=70)	chr1: 161008127-161008977	−12.6%	0.092	*F11R*	0	5’ UTR
	Ozone (O_3)						
	All (n=124)	*no significant DMRs identified*					
	Female (n=54)	*no significant DMRs identified*					
	Male (n=70)	chr6: 36515676-36515856	−0.7%	0.082	STK38	429	Promoter

Note: chr, chromosome; FWER, family-wise error rate, M, DNA methylation level

[a]Average difference in DNA methylation across the differentially methylated region between samples in the highest and lowest quartiles of exposure; reported as a percent.

[b]Family wise error rate, obtained via 1000 permutations, representing the proportion of null regions that are longer and have a greater average difference in DNA methylation than the exposure-associated candidate region

[c]Distance, in base pairs, from the exposure-associated differentially methylated region to the nearest transcriptional start site