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Prenatal Particulate Air Pollution and DNA Methylation in Newborns: An Epigenome-Wide Meta-Analysis

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Background: Prenatal exposure to air pollution has been associated with childhood respiratory disease and other adverse outcomes. Epigenetics is a suggested link between exposures and health outcomes.

Objectives: We aimed to investigate associations between prenatal exposure to particulate matter (PM) with diameter <10 (PM_{10}) or <2.5 μm ($PM_{2.5}$) and DNA methylation in newborns and children.

Methods: We meta-analyzed associations between exposure to PM_{10} ($n = 1{,}949$) and $PM_{2.5}$ ($n = 1{,}551$) at maternal home addresses during pregnancy and newborn DNA methylation assessed by Illumina Infinium HumanMethylation450K BeadChip in nine European and American studies, with replication in 688 independent newborns and look-up analyses in 2,118 older children. We used two approaches, one focusing on single cytosine-phosphate-guanine (CpG) sites and another on differentially methylated regions (DMRs). We also related PM exposures to blood mRNA expression.

Results: Six CpGs were significantly associated [false discovery rate (FDR) <0.05] with prenatal PM_{10} and 14 with $PM_{2.5}$ exposure. Two of the PM_{10}-related CpGs mapped to *FAM13A* (cg00905156) and *NOTCH4* (cg06849931) previously associated with lung function and asthma. Although these associations did not replicate in the smaller newborn sample, both CpGs were significant ($p < 0.05$) in 7- to 9-y-olds. For cg06849931, however, the direction of the association was inconsistent. Concurrent PM_{10} exposure was associated with a significantly higher *NOTCH4* expression at age 16 y. We also identified several DMRs associated with either prenatal PM_{10} and or $PM_{2.5}$ exposure, of which two PM_{10}-related DMRs, including *H19* and *MARCH11*, replicated in newborns.

Conclusions: Several differentially methylated CpGs and DMRs associated with prenatal PM exposure were identified in newborns, with annotation to genes previously implicated in lung-related outcomes. https://doi.org/10.1289/EHP4522

Introduction

Many studies have reported adverse health effects of prenatal air pollution exposure in children, including adverse pregnancy outcomes, reduced lung growth, and increased risks of respiratory morbidity (Lamichhane et al. 2015; Korten et al. 2017; Horne et al. 2018). Findings from experimental models suggest that oxidative stress, inflammation, and mitochondrial dysfunction may contribute to health effects of particulate exposure, but our understanding of the involved mechanisms remains limited (Cassee et al. 2013; Niranjan and Thakur 2017). Recent studies demonstrate that environmental exposures may induce epigenetic modifications and that these changes can have long-lasting effects on gene expression and cell function (Desai et al. 2017; Gref et al. 2017). DNA methylation, the most studied epigenetic mechanism, entails cytosine modification with a methyl group at positions in DNA where a cytosine is located next to a guanine, a cytosine-phosphate-guanine (CpG) site. The crucial role of methylation in maintaining genomic stability and regulation of gene function makes it a potential mechanism by which environmental exposures contribute to the etiology of complex diseases.

Prenatal life is an important window of susceptibility to adverse effects of environmental hazards. *In utero* exposures may lead to epigenetic changes that influence fetal development and contribute to health outcomes throughout the life course (Barouki et al. 2018). Studies on prenatal exposures to cigarette smoke and traffic-related air pollution reported associations with modifications of the offspring epigenome (Joubert et al. 2016; Gruzieva et al. 2017). The majority of published studies investigated variability of DNA methylation in relation to air pollution either globally (i.e., overall methylation state of the genome) (Plusquin et al. 2017) or applying candidate-gene approaches (Somineni et al. 2016; Hew et al. 2015), but comprehensive evaluations of genome-wide DNA methylation patterns in children are limited (Breton et al. 2016; Gruzieva et al. 2017; Plusquin et al. 2018).

Epigenome-wide association studies (EWAS) of particulate air pollution exposure have so far been based almost exclusively on adult populations with inconclusive results. Epigenome-wide association studies of short-term exposure to particulate matter (PM) with an aerodynamic diameter of <2.5 μm ($PM_{2.5}$) reported associations with DNA methylation within genes involved in protein kinase and NFkB pathways (Jiang et al. 2014), as well as oxidative stress (Panni et al. 2016), although no robust associations could be demonstrated with long-term particulate exposure (Plusquin et al. 2017). We have previously found epigenome-wide cord blood DNA methylation differences in several mitochondria-related genes in relation to prenatal exposure to nitrogen dioxide, a marker of traffic-derived combustion pollutants (Gruzieva et al. 2017).

Earlier studies have focused on individual differentially methylated CpGs rather than differentially methylated regions (DMRs) (Breton et al. 2016; Gruzieva et al. 2017; Panni et al. 2016). DMR analysis is a statistically more powerful approach for detecting associations with exposures or health outcomes, as it uses the patterns of correlation between nearby CpGs to take advantage of the epigenomic structure (Pedersen et al. 2012; Peters et al. 2015). For the present study, we meta-analyzed genome-wide DNA methylation data in newborns in relation to maternal exposure to PM during pregnancy to identify both individual CpGs and regions of differential methylation. Furthermore, the associations found between maternal exposure to PM and cord blood DNA methylation were examined in independent data sets of newborn and older children. We also examined differences in peripheral blood gene expression for identified genes in relation to prenatal [in newborns from the Early Autism Risk Longitudinal Investigation (EARLI) cohort, $n = 119$] and current air pollution exposure [in 16-y-olds from the Barn, Allergi, Miljö, Stockholm och Epidemiologi (BAMSE) cohort in Sweden (titled Children, Allergy, Milieu, Stockholm, Epidemiology in English), $n = 244$].

Methods

Detailed information about each of the study cohorts in this analysis, including recruitment and eligibility; information about methods for measuring DMA methylation and gene expression, including quality control and normalization procedures; and detailed information about air pollution exposure estimation, are provided in Supplemental

Material. Average concentrations of PM_{10} and $PM_{2.5}$ throughout pregnancy were estimated at maternal home addresses through land-use regression (LUR) or equivalent models.

Discovery Study Population

A total of nine European and American studies participating in the Pregnancy and Childhood Epigenetics consortium (PACE) (Felix et al. 2017) were included in the discovery meta-analysis of particulate air pollution exposure during pregnancy and newborn DNA methylation (total $N = 2{,}411$): INfancia y Medio Ambiente (INMA), Generation R, Southern California Children's Health Study (CHS), Early Autism Risk Longitudinal Investigation (EARLI), the PRogramming of Intergenerational Stress Mechanisms (PRISM), Project Viva, Environmental Influences on Early Ageing (ENVIR*ON*AGE), Rhea Mother and Child Cohort in Crete, Greece (Rhea), and Piccolipiù (Table 1).

Replication and Look-Up Study Populations

We performed a replication analysis of the PM_{10}-related FDR-significant findings in a separate sample of newborns ($n = 688$) from the ALSPAC project (Relton et al. 2015). A look-up association analysis of the newborn findings at older ages was based on three independent samples of 7- to 9-y-olds: *a*) Mechanisms of the Development of ALLergy (MeDALL) comprising a pooled sample from two cohorts with uniform methylation measurements: BAMSE (Sweden) and Prevention and Incidence of Asthma and Mite Allergy (PIAMA; Netherlands), combined with an independent sample from the BAMSE cohort, BAMSE Epigene (total $N = 692$) (Xu et al. 2018); *b*) Human Early Life Exposome (HELIX), a pooled sample from four cohorts (total $N = 525$) (Vrijheid et al. 2014): Norwegian Mother and Child Cohort (MoBa), Etude de cohorte généraliste, menée en France sur les Déterminants pré et post natals précoces du développement psychomoteur et de la santé de l'Enfant (EDEN), Kaunas Cohort, Lithuania (KAUNAS), and Born in Bradford (BiB), Bradford, UK; *c*) Avon Longitudinal Study of Parents and Children (ALSPAC), UK ($n = 901$); as well as on two samples of 15- to 16-y-olds: BAMSE ($n = 198$) and ALSPAC ($n = 903$). Consent for blood sampling was obtained from all parents. Ethical approval for each study was granted by local institutional review boards.

Statistical Analyses

Cohort-Specific Analyses. For the cohort-specific analyses untransformed normalized methylation, beta values (β-values) were used. The β value is a continuous variable ranging between 0 and 1, representing the ratio of the intensity of the methylated-probe signal to the total locus signal intensity. A β-value of 0 corresponds to no methylation, and a value of 1 corresponds to 100% methylation at the specific CpG site measured. All included samples were analyzed on a cohort level, except the pooled HELIX study and the pooled MeDALL study with coordinated methylation measurements, as well as air pollution exposure assessment according to a harmonized protocol.

First, we examined the associations between exposure to PM and methylation levels across the genome in each cohort separately using multiple robust linear regression [rlm in the In functional analysis of expression data R package (version 3.3.2; R Core Team)] to account for potential outliers and heteroscedasticity in the data (Fox and Weisberg 2011). All analyses were adjusted for an *a priori* selected panel of covariates: child's sex, maternal smoking ever during pregnancy (yes/no), cohort-specific batch indicator (s), and ancestry (in CHS). In addition, age at biosampling, municipality at birth (in BAMSE), and cohort indicator (in the pooled

Table 1. Basic characteristics of cohorts included in the discovery EWAS meta-analysis.

STUDY	Country	Enrollment period	Total *N* enrolled	Selection criteria for EWAS	Air pollution exposure assessment	DNA methylation measurement	Study reference (PMID)	Study website
INMA	Spain	1997–2008	3768	available DNA from one of the subcohorts (Sabadell)	LUR	Illumina 450K	21471022	http://www.proyectoinma.org/
Generation R	Netherlands	2002–2006	9901	European, complete follow-up	LUR	Illumina 450K	23086283, 25527369	www.generationr.nl
CHS	USA	1995–1997	5341	non-Hispanic white/Hispanic white	Outdoor air pollution monitoring stations in each of the study communities	Illumina 450K	16675435, 22896588	https://healthstudy.usc.edu/index.php
EARLI	USA	2009–2012	232	NA	used inverse distance-squared weighting	Illumina 450K	22958474	http://www.earlistudy.org/
PRISM	USA	2012–2014	592	Random sample	hybrid land use regression and satellite-based model	Illumina 450K	24476840, 25328835	NA
Project Viva	USA	1999–2003	2128	Available cord blood or early/mid-childhood blood sample	hybrid land use regression and satellite-based model	Illumina 450K	24639442	https://www.hms.harvard.edu/viva/index.html
ENVIR*ON*AGE	Belgium	2010–2016	1210	Random sample	spatial-temporal interpolation method	Illumina 450K	23742113	www.limburgsgeboortecohort.be
Piccolipiù	Italy	2011–2015	3338	Participants resident in the municipality of Turin with enough stored biological material and with 24-month follow-up data	LUR	Illumina 450K	24506846	www.piccolipiu.it
Rhea	Greece	2007–2008	1500	Random sample	LUR	Illumina 450K	19713286	www.rhea.gr

MeDALL and HELIX sample sets) were included in the analyses of the older children. To account for potential differences in DNA methylation that may arise from variability of cell composition in whole blood (Reinius et al. 2012), we estimated cell type composition in cord blood using a reference panel of cells isolated from cord blood (leukocytes and nucleated red blood cells) (Bakulski et al. 2016), and in the older children using an adult reference panel (Reinius et al. 2012), applying the estimateCellCounts function in the minfi Bioconductor package in R (Jaffe and Irizarry 2014). We adjusted for cell composition by including these estimated cell type fractions as covariates in the multivariable linear regression.

Air pollution concentrations were entered as continuous variables without transformation. The results are presented as difference in methylation β-valueper increase in average interquartile range (IQR) of PM_{10} and $PM_{2.5}$ exposure levels across the cohorts corresponding to 5.6 and $2.0\,\mu g/m^3$, respectively.

Meta-Analyses. A total of 473,723 and 473,680 CpGs were included in the meta-analysis of PM_{10} and $PM_{2.5}$ results, respectively, after quality control filtering, as well as exclusion of probes that mapped to the X ($n = 11{,}232$) or Y ($n = 416$) chromosomes. Cohort-specific results of the cord blood EWAS were subsequently meta-analyzed using fixed-effects inverse variance weighting in version 2011-03-25, METAL (http://www.sph.umich.edu/csg/abecasis/metal/) (Willer et al. 2010). We used the false discovery rate (FDR, $p < 0.05$ for significance) procedure to account for multiple testing (Strimmer 2008). For replication and look-up analyses, a nominal $p < 0.05$ was considered statistically significant. DNA methylation sites were annotated based on data provided by Illumina (Bibikova et al. 2011).

DMR Analyses. Differentially methylated regions were identified using two methods available for use with meta-analysis results, comb-p (version 0.32), which identifies DMRs by regional clustering of low p-values from irregularly spaced p-values (Pedersen et al. 2012) and DMRcate (version 1.8.6; https://www.rdocumentation.org/packages/DMRcate), that identifies DMRs from tunable kernel smoothing process of association signals (Peters et al. 2015). Input files for both DMR analyses were our meta-analyzed single-CpG EWAS results on newborns: regression coefficients, standard deviations, uncorrected p-values for DMRcate and uncorrected p-values and chromosomal locations for comb-p. Significant DMRs were defined based on the following criteria: *a*) a DMR should contain more than one probe; *b*) regional information can be combined from probes within 1,000 bp; *c*) the region showed multiple-testing corrected $p < 0.01$ in both methods (Sidak for comb-p and FDR for DMRcate). DMRs detected by both methods were considered significant in our analysis. Input parameters used for the DMR calling in both algorithms are provided in Table S1.

Functional Follow-Up

We investigated whether genes annotated to the significant CpGs were differentially expressed in cord blood in relation to air pollution exposure during pregnancy in the EARLI ($n = 119$) or at the time of biosampling in the BAMSE cohort ($n = 244$) by means of linear regression analysis. Furthermore, we analyzed the association of the FDR-significant CpG methylation with gene expression in *cis* (250 kb window) in 3,075 adults in the Biobank-based Integrative Omics Studies (BIOS) consortium data set (Bonder et al. 2017), and used FDR correction as threshold.

To identify associations between methylation levels and the expression levels of nearby genes (*cis*-expression quantitative trait methylation, *cis*-eQTM), we regressed methylation M-value on gene expression, sex, sampling age, lymphocytes percentage, monocyte percentage, and RNA Flow Cell Number. The inflation of models is corrected by using "bacon" method (van Iterson et al. 2017). We mapped the eQTM in a window of 250 kb around the identified 5,547 CpG sites. For this analysis, we used a total of 3,075 samples for which both methylation and gene expression data were available from four cohorts: Lifelines DEEP, Rotterdam, Leiden Longevity, and Netherlands Twin Register (NTR).

To identify plausible pathways associated with air pollution exposure, we performed the over-representation analysis based on CpGs significantly associated with prenatal PM exposure in the meta-analysis at an arbitrary cutoff of $p < 10^{-5}$ using ConsensusPathDB (Kamburov et al. 2013), as well as the R Bioconductor package missMethyl (version 1.10.0 gometh function), which performs one-sided hypergeometric tests taking into account and correcting for any bias derived from the use of differing numbers of probes per gene interrogated by the array (Phipson et al. 2016).

Finally, we investigated whether previously reported differentially methylated CpGs related to *in utero* tobacco smoke exposure [6,073 CpGs with FDR-significance (Joubert et al. 2016)] were differentially methylated in relation to prenatal PM exposure. We performed Fisher's exact test for overrepresentation of smoking-related CpGs among nominally significant PM-related CpGs.

We additionally examined whether our FDR-significant CpGs overlapped with the list of potentially polymorphic and cross-reactive probes provided by Chen et al. (Chen et al. 2013), and applied the dip test (Hartigan and Hartigan 1985) for two overlapping CpGs to test for nonunimodal DNA methylation distribution using an independent publicly available data set of cord blood DNA methylation samples (Barrett et al. 2013; Rojas et al. 2015).

Results

The baseline characteristics of the study populations are presented in Table 1 (and Table S2 in the online data supplement). Exposure contrasts were smallest for the PRISM ($PM_{2.5}$ IQR $0.8\,\mu g/m^3$) and RHEA (PM_{10} IQR $2.3\,\mu g/m^3$) cohorts and were highest for the CHS ($PM_{2.5}$ IQR $= 5.0$ and PM_{10} IQR $= 14\,\mu g/m^3$). The discovery meta-analysis of cord blood methylation in relation to prenatal exposure included 1,949 newborns for PM_{10} and 1,551 for $PM_{2.5}$. The difference in sample sizes is due to missing prenatal PM_{10} data for Project Viva and PRISM cohorts, and missing prenatal $PM_{2.5}$ data for the Generation R cohort. Minus $\log_{10}$(p-values) from the combined analysis of CpGs across the genome in cord blood samples are presented in Figure 1. The quantile–quantile plots did not reveal any noteworthy inflation in the distribution of observed p-values ($\lambda = 1.21$ for PM_{10} exposure and 1.37 for $PM_{2.5}$; Figure S1). Study-specific lambdas can be found in Table S3.

Meta-Analyses Findings

We found epigenome-wide significant associations (FDR $p < 0.05$) between PM_{10} exposure and DNA methylation for six CpGs, with higher PM_{10} exposure being associated with an increase in methylation for four CpGs mapping to G*NB2L1*; *SNORD96A*, *FAM13A*, *SRPRB*, and *P4HA2*, and a decrease for two CpGs within *USP4,* and *NOTCH4* (Table 2). Effect sizes were generally small, i.e., 0.1% difference in methylation β-value per IQR $= 5.6\,\mu g/m^3$ increase in prenatal PM_{10} exposure.

We found 14 CpGs significantly associated with prenatal $PM_{2.5}$ using FDR correction, positioned in or near the following genes: *PLXNA4*, *ZNF705A*, 2,5 kb downstream of *C14orf2*, *FNIP1*, *COL22A1*, *TMCO3*, *SFRS8*, 8,1 kb upstream of *NEUROG1*, *MRI1*, *PSG5*, *C7orf50*, 1,1 kb downstream of *MORN1*, *PLAT*, and *ZNF695* (Table 3). The direction of the effect was negative for 11 of these CpGs, and positive for cg16253537 in *FNIP1*, cg01011943 in *PSG5*, and cg00348551 in *C7orf50* in relation to higher $PM_{2.5}$ exposure. The estimates ranged from −0.4% to 0.3% difference in

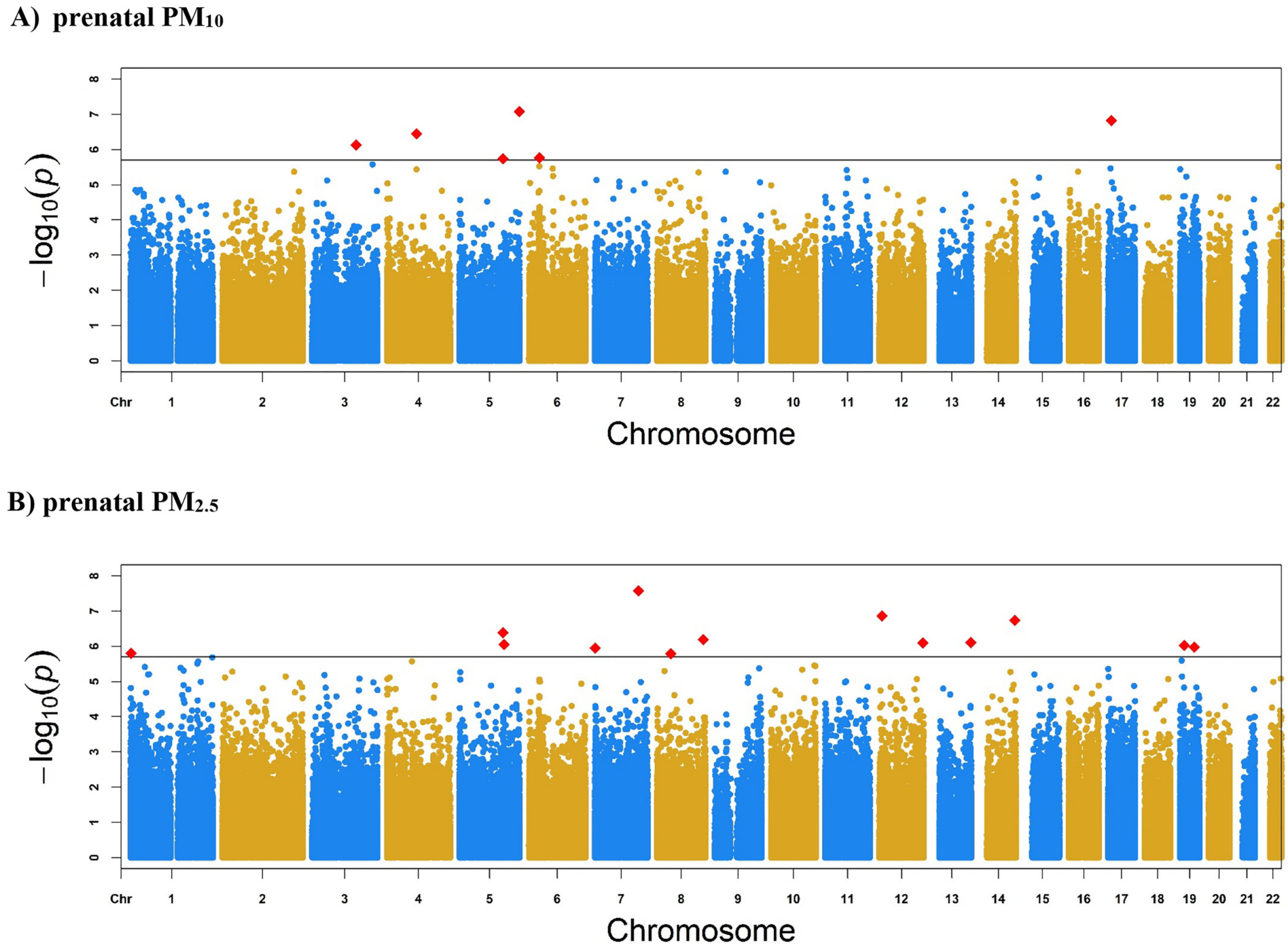

Figure 1. Manhattan plot for epigenome-wide meta-analysis of the association between (A) prenatal PM_{10} ($n = 1{,}949$) and (B) prenatal $PM_{2.5}$ exposure ($n = 1{,}551$) and cord blood DNA methylation. Note: The solid horizontal line corresponds to an FDR rate of 0.05. Manhattan plot for PM_{10}: Six CpGs were considered statistically significant using FDR correction (red squares): cg15082635 in *GNB2L1;SNORD96A*, cg20340716 in *USP43*, cg00905156 in *FAM13A*, cg24127244 in *SRPRB*, cg06849931 in *NOTCH4*, and cg18640183 in *P4HA2*. Manhattan plot for $PM_{2.5}$: Fourteen CpGs were considered statistically significant using FDR correction (red squares): cg16811875 in *PLXNA4*, cg12193649 in *ZNF705A*, cg11886880 2,5 kb upstream of *C14orf2*, cg16253537 in *FNIP1*, cg12044654 in *COL22A1*, cg19120073 in *TMCO3*, cg05479174 in *SFRS8*, cg06846669 8.1 kb downstream of *NEUROG1*, cg23270359 in *MRI1*, cg01011943 in *PSG5*, cg00348551 in *C7orf50*, cg24709511 1,1 kb downstream of *MORN1*, cg22038738 in *PLAT*, and cg02236896 in *ZNF695*.

methylation level per IQR ($IQR = 2\ \mu g/m^3$) increase in prenatal $PM_{2.5}$ exposure.

Two out of the 14 FDR-significant CpGs associated with prenatal $PM_{2.5}$, namely cg12193649 and cg01011943, overlapped with the list of potentially polymorphic and cross-reactive probes provided by Chen et al. (2013). However, results from the dip test applied to those two CpGs did not reveal statistically significant deviation from unimodality ($p = 0.65$ and $p = 0.99$, respectively).

Tests for heterogeneity did not display any major heterogeneity across studies: 8% and 9.9% of the examined PM_{10}- and $PM_{2.5}$-related CpGs, respectively, had heterogeneity $p < 0.05$, and median I^2 statistics for PM_{10} was 0% (ranging between 0–94%) and for $PM_{2.5}$ −5.1% (ranging between 0–88.7%). No significant heterogeneity was found for any of the identified FDR-significant CpGs (p-values for heterogeneity ranging within 0.08–0.81: see forest plots in Figure S2).

Analyses of Differentially Methylated Regions

By applying two different methods for DMR analysis of PM_{10}-related results, we identified 147 significant (FDR $p < 0.01$) DMRs from DMRcate (Table S1) and 12 significant (Sidak $p < 0.01$) DMRs from comb-p (Table S2), including 11 that were significant based on both approaches (Table 4). It is interesting to note that all genome-wide significant individual CpGs identified in the discovery meta-analysis were also found within the 147 DMRs found in DMRcate, with the exception of cg06849931 located in *NOTCH4*.

We also found 272 significant (FDR $p < 0.01$) DMRs from DMRcate (Table S3) and 33 significant (Sidak $p < 0.01$) DMRs from comb-p (Table S4) in relation to prenatal $PM_{2.5}$ exposure, of which 15 overlapped between the two methods (Table S4). Five out of 14 genome-wide significant individual CpGs identified in the discovery meta-analysis were also seen in the DMRs, namely related to genes *C7orf50*, *ZNF705A*, *PLAT*, *PSG5*, and *MRI1*.

Replication and Look-Up Analyses

None of the six FDR-significant CpGs identified as differentially methylated in relation to prenatal PM_{10} in our discovery meta-analysis sample of 1,949 newborns could be replicated in the 688 newborns of the ALSPAC study (Table 2). However, four out of

Table 2. Statistically significant CpGs (FDR $p < 0.05$) associated with IQR increases in prenatal PM_{10} ($5.6\ \mu g/m^3$) exposure and DNA methylation in newborns (discovery meta-analysis), and replication analyses in newborns, children (age 7–9 years) and adolescents (age 15–16 years).

				Discovery: newborns[a]		Replication: newborns	Replication: age 7–9 years			Replication: age 15–16 years	
				($n = 1,949$)		ALSPAC ($n = 688$)	BAMSE EpiGene + MeDALL ($n = 692$)	HELIX ($n = 525$)	ALSPAC ($n = 901$)	BAMSE 16 years ($n = 198$)	ALSPAC 15 years ($n = 903$)
Chr	Position[b]	CpG	Gene[c]	β (*p*-value)	Direction[d]	β (*p*-value)	β (*p*-value)	β (*p*-value)	β (*p*-value)	β (*p*-value)	β (*P*-value)
5	180670110	cg15082635	*GNB2L1; SNORD96A*	0.001 (8.29E-08)	↓↑↓↑↑↑↑	−0.0004 (0.17)	<0.0001 (1.00)	0.0001 (0.75)	0.0006 (0.02)	−0.0001 (0.63)	0.00006 (0.05)
17	9559558	cg20340716	*USP43*	−0.002 (1.50E-07)	↓↑↓↓↓↓↓	0.0011 (0.50)	<0.0001 (0.73)	−0.0013 (0.39)	0.0002 (0.89)	0.0003 (0.19)	0.0004 (0.15)
4	89744363	cg00905156	*FAM13A*	0.001 (3.55E-07)	↓↑X↑↑↑↑	−0.0003 (0.33)	0.0017 (0.03)	−0.0001 (0.84)	0.0004 (0.15)	0.0001 (0.72)	0.00001 (0.90)
3	133524572	cg24127244	*SRPRB*	0.001 (7.33E-07)	↓↑↑↑↑↑↑	−0.00001 (0.97)	<0.0001 (0.77)	0.0002 (0.61)	−0.0003 (0.28)	−0.0002 (0.12)	0.00004 (0.42)
6	32165893	cg06849931	*NOTCH4*	−0.001 (1.72E-06)	↓↓↓↑↑↓↓	0.0003 (0.81)	0.0022 (0.03)	−0.0023 (0.002)	0.0010 (0.33)	0.00002 (0.95)	−0.0002 (0.30)
5	131563610	cg18640183	*P4HA2*	0.001 (1.80E-06)	↑↑↑↑↑↑↑	0.0003 (0.44)	0.0006 (0.61)	0.0009 (0.03)	−0.0001 (0.82)	0.0001 (0.53)	0.00001 (0.86)

Note: β, coefficient for methylation with an IQR increase in prenatal PM_{10} exposure; CHR, chromosome.
[a]Discovery meta-analysis does not include the PRISM or Project Viva cohorts due to missing prenatal PM_{10} data.
[b]Chromosomal position based on NCBI human reference genome assembly Build 37.
[c]UCSC annotated gene.
[d]Direction of methylation for each cohort included in the analysis (INMA, Generation R, CHS, ENVIR*ON*AGE, Rhea, Piccolipiù, EARLI): ↑ = increased methylation, ↓ = decreased methylation, X = not available.

these six CpGs showed significance later in childhood in associations with prenatal PM_{10} exposure; cg00905156 (*FAM13A*) and cg06849931 (*NOTCH4*) showed increased methylation in relation to PM_{10} exposure during pregnancy in the combined BAMSE Epigene and MeDALL samples ($n = 692$) of 7- to 9-y-olds ($p = 0.03$), although the direction of association for cg06849931 was opposite to the one in the discovery analysis (Table 2). Furthermore, cg06849931 was also differentially methylated in the HELIX study ($p = 0.002$), along with cg18640183 (*P4HA2*) ($p = 0.03$), both demonstrating the same direction of association as those in the discovery meta-analysis. In addition, cg15082635 (*GNB2L1*; *SNORD96A*) was also nominally significant in 7-to 9-y-olds from the ALSPAC study with the same direction of association ($p = 0.02$). None of these six associations was present in adolescents from the BAMSE ($n = 198$) and ALSPAC ($n = 903$) studies ($p > 0.05$). Children's concurrent PM_{10} exposure at the time of biosampling was not significantly associated with any of these six CpGs ($p > 0.05$; see Table S5).

Among the 14 epigenome-wide significant $PM_{2.5}$-associated CpGs in newborns, none appeared to be statistically significant in children and adolescents, apart from cg23270359 (*MRI1*), which was significant in the HELIX sample ($p = 0.01$), although the direction of association was opposite to that in the discovery meta-analysis (Table 3).

Two significant gene regions from the discovery PM_{10}-related DMR analyses, including genes *H19* and *MARCH11*, were also FDR-significant in analysis of the ALSPAC newborn sample using DMRcate (replication min FDR $p = 9.5 \times 10^{-4}$ and $p = 3.9 \times 10^{-5}$, respectively).

Functional Follow-Up

The top three PM_{10}-related CpGs, including one within the *FAM13A* gene, as well as six out of 14 $PM_{2.5}$-associated CpGs, were significantly associated with gene expression in *cis* in BIOS (Table S6).

In functional analysis of expression data from the newborns in the EARLI cohort ($n = 119$), no significant association of *in utero* PM_{10} exposure with expression of genes annotated to the respective CpG was detected, whereas $PM_{2.5}$ exposure was associated with expression of *ZNF695* [$p < 0.05$, Log fold change (LogFC) = 0.074 per 2-$\mu g/m^3$ increase in exposure; Table 5]. In BAMSE ($n = 244$), current PM_{10} exposure at 16 y was associated with *NOTCH4* (multiple transcripts, lowest $p = 0.0001$, LogFC = 0.05) and *USP43* expression levels in peripheral blood cells ($p < 0.05$, LogFC = 0.05, per 5.6-$\mu g/m^3$ increase; Table 6). Among the $PM_{2.5}$ associated genes, *C7orf50* was significantly differentially expressed in relation to current $PM_{2.5}$ exposure ($p = 0.03$, LogFC = 0.02, per 2-$\mu g/m^3$ increase). Descriptive statistics of expression levels of genes associated with CpG methylation in response to maternal PM_{10} or $PM_{2.5}$ exposure in the EARLI and BAMSE cohorts are provided in Table S7 and Table S8, respectively.

Pathway Analysis

Twenty-eight of 31 unique gene identifiers extracted from the meta-analysis with PM_{10} exposure matched to ConsensusPathDB. Using FDR $p < 0.05$, six enriched pathways were identified including "Notch Signaling Pathway" (genes *NOTCH4* and *DVL2*), "Rho GTPase cycle" (*FAM13A*; *HMHA1*; *VAV2*; and *GMIP*), "Neurotransmitter Release Cycle" (*HSPA8*; and *RIMS1*), and "GABA synthesis, release, reuptake and degradation" (*HSPA8*; and *RIMS1*). In the repeated pathway analysis using gometh function in missMethyl, no statistically significant pathways were found after correction for multiple testing; however, we observed the same top significant pathways as identified by ConsensusPathDB, i.e., related to

Table 3. Statistically significant CpGs (FDR $p < 0.05$) associated with IQR increases in prenatal $PM_{2.5}$ ($2\ \mu g/m^3$) exposure and DNA methylation in newborns (discovery meta-analysis), and replication in children (age 7–9 years) and adolescents (age 16 years).

Discovery meta-analysis						Replication: age 7–9 years		Replication: age 16 years
Prenatal $PM_{2.5}$ ~ newborn methylation						BAMSE EpiGene + MeDALL (n = 692)	HELIX (n = 603)	BAMSE (n = 198)
Chr	Position[b]	CpG	Gene[c]	β (*P*-value)	Direction[d]	β (*P*-value)	β (*P*-value)	β (*P*-value)
7	132192823	cg16811875	*PLXNA4*	−0.003 (2.67E-08)	↓↓↓↓↓↓↓↓	−0.0006 (0.41)	−0.00085 (0.19)	0.0001 (0.86)
12	8324628	cg12193649	*ZNF705A*	−0.004 (1.37E-07)	↓↑↓↓ XXXX	X	−0.00036 (0.76)	X
14	104376135	cg11886880	2,5 kb down *C14orf2*	−0.001 (1.81E-07)	↓↓ X↑↓↑↑↓	<0.0001 (0.78)	−0.00014 (0.11)	0.0002 (0.50)
5	131132836	cg16253537	*FNIP1*	0.001 (4.10E-07)	↑↑↑↓↑↑↑↑	−0.0004 (0.37)	0.00008 (0.43)	0.0001 (0.63)
8	139890342	cg12044654	*COL22A1*	−0.001 (6.42E-07)	↓↓↓↑↑↓↓↓	0.0004 (0.46)	−0.00033 (0.07)	0.0001 (0.67)
13	114165365	cg19120073	*TMCO3*	−0.001 (7.77E-07)	↓↓↓↓↑↓↓↓	<0.0001 (0.87)	−0.00013 (0.39)	−0.0004 (0.23)
12	132239000	cg05479174	*SFRS8*	−0.001 (7.99E-07)	↓↓↓↑↑↑↑↓	0.0002 (0.87)	−0.00003 (0.93)	0.0005 (0.44)
5	134879739	cg06846669	8,1 kb up *NEUROG1*	−0.002 (8.92E-07)	↓↓↓↓↓↓↓↓	−0.0002 (0.59)	0.00007 (0.77)	−0.0001 (0.88)
19	13875381	cg23270359	*MRI1*	−0.001 (9.43E-07)	↓↓↓↓↓↓↓↓	<0.0001 (0.84)	0.00035 (0.01)	−0.0001 (0.71)
19	43690622	cg01011943	*PSG5*	0.003 (1.05E-06)	↑↑↑↓ XXXX	X	−0.00008 (0.93)	X
7	1177965	cg00348551	*C7orf50*	0.001 (1.13E-06)	↑↑ X↓XXX↑	0.0008 (0.13)	0.00010 (0.51)	−0.0004 (0.32)
1	2251570	cg24709511	1,1 kb down *MORN1*	−0.001 (1.57E-06)	↓↓↓↑↓↑↓↓	−0.0004 (0.50)	−0.00020 (0.30)	0.0004 (0.23)
8	42064673	cg22038738	*PLAT*	−0.004 (1.61E-06)	↓↓↓↓↓↑↓↓	0.0008 (0.39)	−0.00079 (0.51)	0.0006 (0.32)
1	247169036	cg02236896	*ZNF695*	−0.003 (2.05E-06)	↓↓ X↑↓↓↓↓	−0.0012 (0.34)	−0.00055 (0.24)	−0.0003 (0.64)

Note: β, coefficient for methylation with an IQR increase in prenatal $PM_{2.5}$ exposure; CHR, chromosome.
[a]Discovery meta-analysis does not include the Generation R cohort due to missing prenatal $PM_{2.5}$ data.
[b]Chromosomal position based on NCBI human reference genome assembly Build 37.
[c]UCSC annotated gene.
[d]Direction of methylation for each cohort included in the analysis (INMA, Project Viva, CHS, PRISM, ENVIR*ON*AGE, Rhea, Piccolipiù, EARLI): ↑ = increased methylation, ↓ = decreased methylation, X = not available.

regulation of GTPase activity (Table S9). No significantly enriched pathways were identified for $PM_{2.5}$.

Candidate-Gene Analysis of Smoking-Related CpGs

Out of 6,073 FDR-significant CpGs previously reported in relation to maternal smoking exposure (Joubert et al. 2016), 359 showed nominal significance ($p < 0.05$) with prenatal PM_{10} and 390 with $PM_{2.5}$ exposure, which is not more than expected by chance (Fisher's exact test nonsignificant for overrepresentation of smoking-related CpGs among nominally significant PM-related CpGs). None of the genome-wide significant CpGs identified in our meta-analyses with PM_{10} and $PM_{2.5}$ were among the 6,073 smoking-related sites.

Discussion

In this large-scale epigenome-wide meta-analysis evaluating the association between prenatal particulate air pollution exposure and DNA methylation in newborns, we found significant associations for PM_{10} and $PM_{2.5}$ exposure during pregnancy with methylation differences in several genes of relevance for respiratory health, such as *FAM13A* and *NOTCH4*. Some of these associations were also seen in the older children. We also identified a number of unique DMRs associated with PM exposure by applying two independent methodologies. The observed differentially methylated genes in the newborn discovery data set represent novel associations in the context of air pollution exposure. One of the top significant hits, cg00905156, localizes in the gene *FAM13A*, which has been identified in multiple genome-wide association studies (GWAS) of pulmonary function and the related phenotype of COPD (Hobbs et al. 2017; Hancock et al. 2010). Research has shown that *FAM13A* interferes with the Wnt pathway, inducing β-catenin degradation, which in turn may affect lung repair (Jiang et al. 2016). *In vitro* studies have also demonstrated differences in respiratory epithelial cell expression of *FAM13A* during differentiation into pulmonary type II cells (Wade et al. 2006).

Another significant CpG site, cg06849931, is located in the *NOTCH4* gene, which has been identified in GWAS as a genetic marker of asthma-related traits (Li et al. 2013). Recently, an

Table 4. DMRs in Relation to prenatal PM_{10} exposure that overlap between DMRcate and comb-p methods.

DMRcate							Comb-p			
Chr	Start	End	No. of probes	Max **βFC**[a]	*p*-Value[b]	Gene[c]	Start	End	No. of probes	*p*-Value[d]
7	27169674	27171528	25	1.58E-03	9.20E-10	*HOXA4*	27169957	27171052	17	2.75E-12
11	2019730	2021243	29	1.45E-03	6.75E-06	*H19*	2020101	2020418	10	4.30E-04
4	2366103	2367137	7	8.24E-04	6.05E-05	*ZFYVE28*	2366555	2367138	5	1.68E-05
6	31963526	31964754	10	−6.85E-04	6.05E-05	*C4A*	31964193	31964392	5	2.42E-04
1	75198211	75199117	11	4.93E-04	1.25E-04	*CRYZ; TYW3*	75198403	75198842	6	7.01E-03
6	170596856	170598215	7	−1.10E-03	3.26E-04	*DLL1; FAM120B*	170597326	170597589	4	2.96E-03
12	52400530	52401523	8	1.16E-04	5.36E-04	*GRASP*	52400530	52400908	5	3.02E-03
10	3823907	3825031	7	−8.09E-04	6.43E-04	*KLF6*	3824387	3824688	4	7.18E-04
1	1549799	1550886	12	−5.38E-04	8.50E-04	*MIB2*	1550648	1550887	8	6.48E-03
19	3970736	3971417	7	−2.32E-04	1.00E-03	*DAPK3*	3971119	3971418	5	3.60E-04
19	12876846	12877188	4	3.69E-03	2.65E-03	*HOOK2*	12876846	12877189	4	7.51E-04

[a]Fold change in DNA methylation β-value.
[b]Minimum FDR *p*-value for the region.
[c]Annotated gene(s) in the region.
[d]Sidak *p*-value.

Table 5. Associations between PM exposure and gene expression levels in newborn children of the EARLI cohort ($n = 119$).

Chr	Gene	ProbeID	LogFC	p-Value
Prenatal PM_{10}				
4	*FAM13A*	16977925	0.025	0.57
6	*NOTCH4*	17017814	−0.003	0.94
6	*NOTCH4*	17027038	−0.008	0.90
6	*NOTCH4*	17029639	0.063	0.14
6	*NOTCH4*	17034630	−0.015	0.63
6	*NOTCH4*	17037128	0.018	0.51
6	*NOTCH4*	17039839	−0.050	0.19
6	*NOTCH4*	17042335	−0.029	0.38
5	*SNORD96A*	17119456	−0.038	0.19
5	*P4HA2*	16999712	−0.018	0.57
17	*USP43*	16831046	−0.034	0.26
3	*SRPRB*	16945907	−0.039	0.46
Prenatal $PM_{2.5}$				
7	*C7orf50*	17054312	0.001	0.98
19	*ZNF606*	16876074	−0.040	0.21
19	*PSG5*	16872926	−0.003	0.89
1	*ZNF695*	16701484	0.074	0.04
10	*MKX*	16712773	0.022	0.45
2	*CAPN10*	16893222	−0.030	0.15
8	*COL22A1*	17081580	0.008	0.63
12	*ZNF705A*	16747907	−0.040	0.11
5	*FNIP1*	16999631	−0.025	0.27
7	*PLXNA4*	17063005	−0.025	0.17
13	*TMCO3*	16776883	−0.019	0.32
8	*PLAT*	17076726	−0.023	0.26
1	*VANGL2*	16672635	−0.026	0.21
19	*MRI1*	16858849	−0.023	0.26

Note: Results presented per $5.6\,\mu g/m^3$ increase in PM_{10} and $2\,\mu g/m^3$ increase in $PM_{2.5}$ exposure for genes annotated to FDR significant CpGs in the discovery PM_{10} and $PM_{2.5}$ EWAS. LogFC = logarithm fold-change (one unit of the logFCs translates to a two-fold change in expression). Adjusted for sex, maternal smoking during pregnancy, and cell composition.

animal study proposed *Notch4* as a susceptibility gene for ozone-induced lung injury (Verhein et al. 2015). Genome-wide transcriptomic analysis of lung tissue homogenates within the same study suggested that upregulation of NOTCH3 and NOTCH4 receptors may protect against inflammation. Our other observed differentially methylated CpGs reside in *USP43*, *SRPRB, GNB2L1*; *SNORD96A*, and a T_H2 cytokine gene, *P4HA2*. *GNB2L1* and *P4HA2* have previously been suggested as candidate genes associated with the susceptibility and prognosis for lung cancer (Choi et al. 2015; Dong et al. 2012).

We were not able to replicate FDR-significant CpGs using a smaller independent methylation data set of newborns. However, in two out of three independent samples of school-age children, cg06849931 (*NOTCH4)* was found to be significantly differentially methylated in relation to prenatal PM_{10}. The direction of association in one of these two samples was opposite, however. Also, significant differential methylation was observed for CpGs in *FAM13A*, *GNB2L1*, and *SNORD96A*, as well as *P4HA2* in one out of three independent samples of school-age children, with the same direction of association as those in the discovery EWAS. Furthermore, expression of the *NOTCH4* gene in BAMSE participants at 16 years of age was increased in association with concurrent exposure to PM_{10}. Lack of replication in newborns may be attributed to generally weak effects of air pollution exposure that may be difficult to detect in a smaller sample. Furthermore, differences in exposure contrasts should be acknowledged, i.e., wide exposure range in the discovery analysis explained by inclusion of cohorts from areas with different exposure levels in comparison with the replication data set.

We found several significantly differentially methylated CpGs in relation to prenatal $PM_{2.5}$ exposure, all of which were distinct from those related to prenatal PM_{10}. Unfortunately, no independent newborn data set with $PM_{2.5}$ data was available for replication analysis. Look-up analysis in older children age 7–9 y suggested association of differential methylation of cg23270359 located in *MRI1*. Previous studies reported significant association between increased *MRI1* methylation and severe asthma (Wysocki et al. 2015).

Earlier epigenome-wide association studies of long-term particulate exposure in adults failed to demonstrate robust associations (Plusquin et al. 2017; de FC Lichtenfels et al. 2018). This failure to demonstrate robust associations may be partly explained by limited statistical power. In addition to EWAS meta-analysis based on single probes, we also investigated regions of differential methylation. Several significant findings were discovered in relation to PM exposure, and the DMR results partly overlapped between the two methods applied, as well as with the genes identified in the single probe meta-analysis. Two of the PM_{10}-related DMRs comprising *H19* and *MARCH11* genes were also replicated in the independent newborn data set. The *H19* gene is located in an imprinted region of chromosome 11 and is expressed only from the maternally inherited chromosome. Recent evidence suggests that *H19* functions as an oncogene and inhibits the activity of tumor suppressor p53 but also plays an important role in embryonic development and growth control (Chen et al. 2018). DNA methylation levels at the *H19* DMR have also been associated with being small for gestational age (Qian et al. 2016).

Our study is one of the first large-scale studies assessing the association of prenatal PM exposure on the neonatal blood methylome. Recently published EWAS meta-analysis of five cohorts ($n = 1{,}235$) did not show any association of maternal PM_{10} exposure during pregnancy with DNA methylation in cord blood (Plusquin et al. 2018). However, pathway analysis of top hits revealed enriched pathways relating to the GABAergic synapse, as well as NOTCH signaling, which is in line with our results. Analysis based on the CHS demonstrated DNA methylation variability in newborn blood in relation to prenatal exposure to PM_{10}

Table 6. Associations between PM exposure and gene expression levels in 16-y-old children of the BAMSE cohort ($n = 244$).

Chr	Gene	ProbeID	LogFC	p-Value
Concurrent PM_{10}				
6	*NOTCH4*	TC6_mcf_hap5000165.hg.1	0.05	9.52E-05
6	*NOTCH4*	TC6_apd_hap1000098.hg.1	0.06	9.73E-05
6	*NOTCH4*	TC6_mann_hap4000155.hg.1	0.06	9.98E-05
6	*NOTCH4*	TC6_cox_hap2000190.hg.1	0.05	1.03E-04
6	*NOTCH4*	TC6_ssto_hap7000159.hg.1	0.05	1.10E-04
6	*NOTCH4*	TC06001564.hg.1	0.05	1.31E-04
6	*NOTCH4*	TC6_qbl_hap6000179.hg.1	0.05	1.42E-04
17	*USP43*	TC17000146.hg.1	0.05	4.98E-04
4	*FAM13A*	TC04001380.hg.1	−0.01	2.14E-01
3	*SRPRB*	TC03000725.hg.1	−0.01	2.89E-01
Concurrent $PM_{2.5}$				
7	*C7orf50*	TC07001077.hg.1	0.02	0.03
19	*PSG5*	TC19001582.hg.1	0.02	0.06
1	*VANGL2*	TC01001369.hg.1	0.02	0.08
1	*ZNF695*	TC01006392.hg.1	0.01	0.08
8	*COL22A1*	TC08001675.hg.1	0.02	0.10
8	*PLAT*	TC08001175.hg.1	0.02	0.16
19	*MRI1*	TC19000239.hg.1	−0.01	0.17
2	*CAPN10*	TC02005015.hg.1	0.01	0.21
10	*MKX*	TC10001133.hg.1	0.01	0.29
13	*TMCO3*	TC13000425.hg.1	−0.01	0.30
7	*PLXNA4*	TC07001877.hg.1	0.01	0.33
19	*ZNF606*	TC19001910.hg.1	−0.0003	0.96

Note: Results presented per $5.6\,\mu g/m^3$ increase in PM_{10} and $2\,\mu g/m^3$ increase in $PM_{2.5}$ exposure for genes annotated to FDR significant CpGs in the discovery PM_{10} and $PM_{2.5}$ EWAS. LogFC = logarithm fold-change (one unit of the logFCs translates to a two-fold change in expression). Adjusted for sex, maternal smoking during pregnancy, active smoking at the time of biosampling, age at biosampling, municipality at birth, doctor's diagnosis of asthma, and cell composition.

and $PM_{2.5}$, some of which were also associated with cardiorespiratory health outcomes later in childhood, including asthma and elevated blood pressure later in childhood (Breton et al. 2016). We have also recently reported associations of NO_2 exposure during pregnancy with cord blood methylation differences in several genes involved in mitochondria function, and we noted that these associations with *in utero* exposure persisted into early childhood (Gruzieva et al. 2017). We did not, however, observe the same associations with PM exposure in the present study. It remains to be investigated whether those associations we observed with NO_2 are pollutant-specific, or whether lack of overlap between NO_2 and PM-related findings are attributed to difference in the sources of particulate pollution in different cities and locations (Eeftens et al. 2012). This difference in sources and chemical composition of PM may also be responsible for the lack of comparability between the present results with PM_{10} and $PM_{2.5}$ exposures.

Some previous EWASs have identified and replicated extensive exposure-associated epigenetic alterations, for example in relation to exposure to maternal tobacco smoke (Joubert et al. 2016). Not only is particulate air pollution a different type of exposure, but also exposure levels are generally much lower than those related to tobacco smoking, which may explain differences in the magnitude of differential methylation patterns associated with exposure. Furthermore, measurement error in assignment of exposure to maternal smoking during pregnancy is likely much lower than for air pollution. Identifying robust signals at single CpG site level for complex exposures such as long-term air pollution may also require larger sample sizes than available in the present study. In addition, all the study populations were from countries with relatively low ambient levels of particulate air pollution. Inclusion of populations with higher exposures may help identify possible effects on DNA methylation.

This study has some weaknesses. We estimated individual concentrations only for outdoor air pollution at residential addresses, which are not equivalent to personal exposure. Also, due to lack of trimester-specific prenatal exposure data, we were not able to explore the importance of exposure time windows during pregnancy. Participants likely travel to several locations throughout the day and may spend more time at locations other than their residential addresses (e.g., workplaces), which may introduce some misclassification, although most likely nondifferential and thus would generally tend to attenuate the associations. However, ambient PM_{10} and $PM_{2.5}$ levels have been consistently associated with negative health effects in multiple studies, including effects on fetal and neonatal outcomes (Lamichhane et al. 2015). Our analyses included studies based in western Europe and the United States, which have relatively lower air pollution concentrations in comparison with many other places. We should also acknowledge that the study included mainly white populations, and generalizability to other ethnic groups is uncertain.

Although we adjusted our analyses for predefined important covariates, residual confounding cannot be ruled out. Another possible limitation is that we used estimated cell counts in our analyses because measured cell types or single-cell methylation data were not available in all cohorts. However, such estimated cell type adjustment has been shown to be appropriate in epidemiological settings (Kaushal et al. 2017).

Methylation signatures are tissue and cell specific (Bakulski and Fallin 2014), and therefore, selection of relevant tissues and cells is of crucial importance for epigenetic analyses. The majority of previous studies have used peripheral blood cells to examine DNA methylation patterns associated with environmental exposures; however, air pollution exposure has also been associated with DNA methylation and expression changes in placenta (Cai et al. 2017; Saenen et al. 2017; Abraham et al. 2018), and lung epithelial cells (Clifford et al. 2017; Zhou et al. 2015). Clifford et al. reported differential methylation of CpG sites in *HOXA4* in response to diesel exhaust following prior exposure to allergen (Clifford et al. 2017), which was also identified as DMR in our analysis with prenatal PM_{10} exposure. *HOXA4* belongs to the family of Hox genes encoding homeodomain transcription factors that determine cell and tissue identities in the developing embryo and patterning of the developing mouse lung (Packer et al. 2000).

In conclusion, our epigenome-wide meta-analysis provides suggestive evidence of newborn methylation differences in several genes with relevance for airway disease, in relation to prenatal particulate air pollution exposure. Some of these associations were also observed later in childhood. Our results also point to the importance of considering the combined effect of nearby CpGs as DMRs when evaluating the impact of exposure on DNA methylation. Further studies are warranted to establish whether this epigenetic variability could potentially explain the influence of ambient air pollution on development of respiratory outcomes.

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