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Residential $PM_{2.5}$ exposure and the nasal methylome in children

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106505.

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Abstract

Rationale: $PM_{2.5}$-induced adverse effects on respiratory health may be driven by epigenetic modifications in airway cells. The potential impact of exposure duration on epigenetic alterations in the airways is not yet known.

Objectives: We aimed to study associations of fine particulate matter $PM_{2.5}$ exposure with DNA methylation in nasal cells.

Methods: We conducted nasal epigenome-wide association analyses within 503 children from Project Viva (mean age 12.9 y), and examined various exposure durations (1-day, 1-week, 1-month, 3-months and 1-year) prior to nasal sampling. We used residential addresses to estimate average daily $PM_{2.5}$ at 1 km resolution. We collected nasal swabs from the anterior nares and measured DNA methylation (DNAm) using the Illumina MethylationEPIC BeadChip. We tested 719,075 high quality autosomal CpGs using CpG-by-CpG and regional DNAm analyses controlling for multiple comparisons, and adjusted for maternal education, household smokers, child sex, race/ethnicity, BMI z-score, age, season at sample collection and cell-type heterogeneity. We further corrected for bias and genomic inflation. We tested for replication in a cohort from the Netherlands (PIAMA).

Results: In adjusted analyses, we found 362 CpGs associated with 1-year $PM_{2.5}$ (FDR < 0.05), 20 CpGs passing Bonferroni correction ($P < 7.0x10^{-8}$) and 10 Differentially Methylated Regions (DMRs). In 445 PIAMA participants (mean age 16.3 years) 11 of 203 available CpGs replicated at $P < 0.05$. We observed differential DNAm at/near genes implicated in cell cycle, immune and inflammatory responses. There were no CpGs or regions associated with $PM_{2.5}$ levels at 1-day, 1-week, or 1-month prior to sample collection, although 2 CpGs were associated with past 3-month $PM_{2.5}$.

Conclusion: We observed wide-spread DNAm variability associated with average past year $PM_{2.5}$ exposure but we did not detect associations with shorter-term exposure. Our results suggest that nasal DNAm marks reflect chronic air pollution exposure.

1. Introduction

Air pollution exposures are known to affect incidence and severity of multiple chronic health conditions, (Garcia et al., 2019; Schraufnagel et al., 2019) and in particular may exacerbate respiratory symptoms in asthma (Orellano et al., 2017) and chronic obstructive pulmonary disease (Hansel et al., 2016). Respirable air pollution particles of 2.5 μm diameter or less ($PM_{2.5}$) have been shown to disrupt the airway epithelial barrier (Zhao et al., 2018), enhance responses to inhaled allergens, (He et al., 2017) and promote oxidative stress response pathways (Feng et al., 2016). The specific mechanisms of action for $PM_{2.5}$-induced adverse

effects are not entirely elucidated, (Rider and Carlsten, 2019) and may potentially be driven by epigenetic modifications, which in turn alter gene expression levels, potentially shifting both local immune responses and epithelial barrier function in the airways.

The majority of studies that have examined air pollution exposures and their associations with epigenetic modifications have focused on DNA methylation (DNAm) patterns in cord blood (Gruzieva et al., 2019) or blood leukocytes (Bind et al., 2014, 2015; Prunicki et al., 2018). These studies (Bind et al., 2014; Gruzieva et al., 2019; Prunicki et al., 2018) have shown epigenetic modification of immune signaling and inflammatory genes, specifically differential DNAm in or near *FOXP3*, *IL-10, IL-6, IFN-γ*, *ICAM*, and *NOTCH4* which may be distinct from localized responses in the airways. Epigenetic analyses in airway cells may identify pathways relevant to air pollution-induced damage and repair responses in the tissue that is first to encounter these exposures, and may also shed light on the underlying mechanisms in air pollution induced exacerbation of existing respiratory disease. Nasal epithelial cells are the most relevant tissue type for research on allergic rhinitis phenotypes and upper airway irritant responses. The nasal epithelium can also serve as a useful surrogate for cells in the lower airway, given the similarities with respect to airway epithelial cell markers (cytokeratin 19, CD44), and the positive correlation between epithelial cell cytokine production (including IL-6, RANTES, vascular endothelial growth factor, monocyte chemoattractant protein-1,and MMP-9) in response to stimulation with IL-1Beta, TNF-alpha, and IL-13 for these two tissue types (McDougall et al., 2008; Thavagnanam et al., 2014). Thus far, studies of air pollution exposures and epigenetic alterations in airway epithelial cells have been very limited. One cross-sectional study among 24 participants identified nasal cell DNAm changes in *TET1* (a gene for a dioxygenase that plays an active role in demethylation of DNA) associated with traffic-related air pollution (TRAP) (Somineni et al., 2016).

In this work our aim was to determine the association of residence-specific air pollutant exposures ($PM_{2.5}$) with genome-wide DNA methylation in the nasal epithelium of children. We conducted our study in 503 participants in Project Viva, (Oken et al., 2015) a pre-birth cohort study, and examined the potential relationship between $PM_{2.5}$ and nasal epithelium DNAm at over 700,000 CpG sites. We replicated our findings in an independent cohort from the Netherlands, the PIAMA birth cohort (N = 445) (Wijga et al., 2014). We hypothesized that prior $PM_{2.5}$ exposure would be associated with altered nasal epithelium DNAm in oxidative stress and epithelial barrier function genes, and we studied various exposure time windows to determine the potential relevance of exposure duration on epigenetic modifications. We also determined whether DNAm patterns associated with $PM_{2.5}$, an exposure known to exacerbate asthma, overlap with the altered nasal DNAm patterns we previously observed with asthma and airway inflammation phenotypes (Cardenas et al., 2019).

2. Methods

2.1. See supplement for additional details on all methods.

Study Populations.—Study participants were enrolled in Project Viva, a prospective pre-birth cohort study (Oken et al., 2015). Of the total 2,128 live births, 1,038 children

were re-contacted at mean 12.9y (11.9 to 15.4y) and attended an early-teen in-person visit, of whom 547 provided consent for nasal swab sample collection. Of these, 503 also had residential specific $PM_{2.5}$ exposure assessment. All study protocols were approved by the Institutional Review Board of Harvard Pilgrim Health Care.

$PM_{2.5}$ Exposure Assessment.—We estimated ambient $PM_{2.5}$ exposure at each participant's residential address using a spatio-temporal model for the Northeastern USA, as previously described (Kloog et al., 2014; Rice et al., 2019). The $PM_{2.5}$ model utilized satellite-based Aerosol Optical Depth data, retrieved using the Multi- Angle Implementation of Atmospheric Correction algorithm at 1 × 1 km resolution, and ground-level daily $PM_{2.5}$ mass measurements, land use terms and meteorological covariates to estimate daily $PM_{2.5}$ at 200 × 200 m resolution. Predictions from this model had an excellent mean out-of-sample R^2 (0.88) and excellent fit of predictions when compared with withheld measurements (slope = 0.99).

DNAm Measurements.—Trained research assistants collected nasal swabs from the anterior nares. Sterile cotton swabs used for sampling were placed in DNA lysis buffer (Promega, Madison, WI, USA) after collection. DNA was isolated using the Maxwell 16 Buccal Swab LEV DNA Purification Kit following the manufacturer's instructions (Promega, Madison, WI, USA). Swab samples from the anterior nares have been previously demonstrated to yield respiratory epithelial cells (Lai et al., 2015). We analyzed extracted DNA using the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA) to obtain epigenome-wide DNAm measurements.

Statistical Analyses.—Among 503 participants with high quality DNAm data eligible for analyses, we performed epigenome-wide association analyses (EWAS) by fitting linear regression models using *limma* with moderated test-statistics using an empirical Bayes estimation (pollution levels were modeled as predictors of DNAm) (Smyth, 2004). We adjusted for variables selected a priori and based on PCA plots in EWAS models: child race/ethnicity, sex, age at sample collection in days, age and sex-specific BMI *z*-score using US national reference data; smokers currently living in the house, sine and cosine of season of sample collection, and maternal education in pregnancy. To account for cell type heterogeneity, we used a bioinformatic method (ReFACTor), adjusting for the first 10 ReFACTor PCs in our analyses (Rahmani et al., 2017). To remove residual inflation and reduce the influence of potential unmeasured confounders, we used the R package bacon, which constructs an empirical null distribution using a Gibbs sampling algorithm, to adjust our EWAS results (van Iterson et al., 2017). We modeled DNA methylation as M values (methylation intensity) but include the beta value (proportion methylated) coefficients here for interpretability. Coefficients are expressed as % change in DNAm for a 1 $\mu g/m^3$ increase in $PM_{2.5}$ exposure level. We performed 5 independent EWAS to analyze nasal DNAm in relation to: 1) $PM_{2.5}$ levels during 24 h prior to nasal sample collection 2) 7-day average $PM_{2.5}$ levels prior to nasal sample collection 3) prior 30-day average $PM_{2.5}$ levels 4) prior 90-day average $PM_{2.5}$ levels 5) prior 365-day (past year) average $PM_{2.5}$ levels. The EWAS was controlled for the false discovery rate (FDR) at 5% and for the familywise error rate using a Bonferroni correction ($P < 6.95x10^{-8}$). In addition to the EWAS on

all available participants (as described above), we also performed a sensitivity analysis removing individuals with current asthma.

After conducting individual CpG by CpG analyses, we performed regional DNAm analyses using DMRff (Suderman et al., 2018) to identify differentially methylated regions (DMRs) associated with $PM_{2.5}$ exposure.

We also conducted pathway analyses of differentially methylated individual CpGs using the R package MissMethyl (Phipson et al., 2015). For pathway analyses, CpGs with FDR < 0.05 were examined for KEGG pathway enrichment.

External Replication Analysis.—We sought to replicate our findings in PIAMA (Wijga et al., 2014)(Prevention and Incidence of Asthma and Mite Allergy), a birth cohort study of children born in 1996–1997 in the Netherlands. In PIAMA participants (age 16 years), nasal epithelial cells were collected by nasal brushings in two study centers (Groningen and Utrecht) (Xu et al., 2018). The lateral area underneath the inferior turbinate was sampled using a Cytosoft brush (CP-5B, Cyto-Pak). Further details on nasal sampling and sample preparation have been previously described (Xu et al., 2018). DNA extracted from nasal brushes was analyzed using the Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA, USA). DNA methylation data were pre-processed in R with the Bioconductor package *minfi* (Aryee et al., 2014) using the original IDAT files, and quality control procedures were performed as previously described (Qi et al., 2020). Exposure to $PM_{2.5}$ was assessed as the annual average at the home address at the time of the age 16 medical examination using land-use regression models that have been described elsewhere (Eeftens et al., 2012). The replication analyses included 445 participants. Analyses were performed with stratification by PIAMA study center, and inverse variance–weighted fixed-effects *meta*-analyses on results from the two PIAMA centers were performed with METAL. Covariate adjustment in replication analyses included age, sex, batch, secondhand smoking, maternal education, season, and 3 surrogate variables. The 3 surrogate variables were added to adjust for unknown confounders, including cell type.

3. Results

Participant Characteristics.

We assessed the nasal methylome in Project Viva participants who were in early adolescence (mean age 12.9 years, SD 0.65) (Table 1). Half of the participants were female (50.1%), and the majority were of white race/ethnicity (68.6%) with representation from other racial/ethnic groups (15.7% Black, 3.6% Hispanic, 3.0% Asian, 9.1% more than one race). Nasal swab sampling was conducted in all seasons (about one third of samples were collected in summer, while 19–26% were collected in fall, winter and spring). On average, participants were exposed to $PM_{2.5}$ below the EPA air quality standard levels. Average past week $PM_{2.5}$ exposure was 7.42 $\mu g/m^3$ (SD 2.00), past month average $PM_{2.5}$ was 7.44 $\mu g/m^3$ (SD 1.53), past 90 day average was 7.50 $\mu g/m^3$ (SD 1.09) and past year average $PM_{2.5}$ exposure was 7.76 $\mu g/m^3$ (SD 0.55). Past year $PM_{2.5}$ showed very low correlations with past day, past 7 day and past month exposures at the same address (Pearson r = 0.03, 0.18 and 0.19,

respectively). Past year $PM_{2.5}$ showed a moderate correlation with past 90 day exposure (0.53). Correlations for all exposures are shown in supplemental table 1.

Global DNA DNAm Variability.

Univariate predictors of global DNAm PCs are shown in Fig. 1. We examined associations of participant characteristics and surrogate variables for cell type (ReFACTor PCs) with the top 30 global DNAm PCs explaining 59% of DNAm variability. As expected, the cell type surrogate variables (PCs on the y-axis of Fig. 1) showed the strongest associations with global DNAm. Season was associated with the first global DNAm PC at $p < 0.01$. Age, race and sex were also associated with some of the top 10 global DNAm PCs. $PM_{2.5}$ exposure (past year and past 90 day levels) was also associated with global DNAm.

$PM_{2.5}$ and DNAm Analyses.

In our EWAS of $PM_{2.5}$ exposure 1-year prior to sample collection, we identified 362 differentially methylated individual CpGs at FDR < 0.05 and 20 CpGs that were statistically significant at the Bonferroni adjusted threshold ($P < 6.95x10^{-8}$) (Table 2). We used the R package bacon to adjust for inflation in our EWAS results. A Manhattan plot of the past year $PM_{2.5}$ exposure EWAS is shown in Fig. 2. Cell type adjustment reduced our FDR significant findings in the 90 day and past year $PM_{2.5}$ exposure models (Supplemental Table 2). After applying bacon, genomic inflation was similar for cell type adjusted models vs. models that did not include cell type ($\lambda = 1.15$ vs. 1.10 for past year $PM_{2.5}$ exposure).

We did not find strong evidence for enrichment based on positional location of CpGs (i.e. CpG position relative to islands) (Supplemental table 3).

The top 20 CpG sites ranked on p-value associated with past year $PM_{2.5}$ exposure and reaching statistical significance after Bonferroni correction are shown in Table 3. Findings included differential methylation of CpGs in solute transport protein genes (*SLC2A9*), epithelial membrane sodium ion transport genes (*SCNN1D*), fibronectin binding domains (*ELFN2*), a phosphoprotein gene associated with T-cell responses (*PAG1*) and a zinc finger protein/ innate immune response gene (*RBCK1*). Other top differentially methylated CpGs were located within genes promoting cell division (*PPP2R5C*), apoptosis (*TMEM214*), DNA repair/carcinogenesis (*LGR6*) and cellular responses to DNA damage (*MACROD2*). Higher previous year $PM_{2.5}$ exposure was associated with lower DNAm of CpG sites in some of the cancer pathway and cell cycle genes in our EWAS results (*TMEM214, LGR6*), while in others DNAm levels were higher with higher $PM_{2.5}$ exposure (*PPP2R5C*). FDR adjusted results (N = 362 sites at FDR < 0.05) are shown in supplemental table 4. These results included additional genes associated with DNA repair/carcinogenesis (*RAD52, UIMC1, WNT7A, GSK3B*), notch signaling (*NOTCH4*), immune response (*VDR, NFKB2, PSTPIP1, TICAM1*), the mTOR pathway (*VDR, ULK1, FGF3*) and MAPK signaling (*TAOK1, CACNA1D, DUSP4, FGF3*).

We also performed a sensitivity analysis with individuals with current asthma removed (N = 375 controls without current asthma were analyzed). For the top differentially methylated CpG sites, comparisons between the original EWAS coefficients versus coefficients with current asthmatics removed showed little change (supplemental table 5).

Our Bonferroni and FDR adjusted findings for past year $PM_{2.5}$ exposure were entirely distinct from the CpG sites identified as associated with asthma in our previous work in Project Viva (Cardenas et al., 2019), however we did identify overlap in the genes annotated to FDR significant CpGs in both the asthma and past year $PM_{2.5}$ exposure EWAS. Although the CpGs themselves were different, both analyses identified sites associated with the following 11 genes: *EPS15L1, NARF, NCOR2, PTPRS, RNF40, TPCN1, LGR6, PTPRE, RIN3, SLC45A4* and *TBC1D22A*. Our previously identified differentially methylated CpG sites for asthma are shown in supplemental table 6 for reference. A sex-stratified EWAS of past year $PM_{2.5}$ exposure and the nasal methlyome did not show sex-specific differences (supplemental table 7).

While the majority of differentially methylated CpGs were detected in our analysis of past year $PM_{2.5}$ exposure, we also detected a minor signal from our cell-type adjusted EWAS of past 90-day $PM_{2.5}$ exposure (2 CpGs were significant at the Bonferroni threshold level, and 9 CpGs that were significant at FDR < 0.05) (Supplemental table 8). One of the CpGs significant at FDR < 0.05 (cg02548780) overlapped with the differentially methylated CpGs associated with past year $PM_{2.5}$ exposure, with consistent direction of association. We did not identify any differentially methylated CpG sites associated with past month, past week or past day $PM_{2.5}$ exposure.

Replication Analysis Results.

A comparison of baseline demographic characteristics of participants in PIAMA and Project Viva is shown in supplemental table 9, and a methods comparison table is shown in supplemental table 10. PIAMA participants (N = 445) were on average 3.4 year older than Project Viva participants, and experienced approximately double the $PM_{2.5}$ exposure levels (mean 16.1 $\mu g/m^3$ (SD 0.7)) as compared to Project Viva (mean 7.76 $\mu g/m^3$ (SD 0.55)). Given that the epigenome was interrogated using a 450 K array in PIAMA (vs. the 850 K array in Project Viva), not all sites were available for replication analysis (10 out of 20 Bonferroni adjusted sites and 203 out of 362 FDR adjusted sites were available for replication analysis in PIAMA). In the replication analysis in PIAMA, we did not observe replication of the 10 available differentially methylated CpGs that met Bonferroni correction in our analysis. However, we did observe replication of 11 out of the 203 CpGs identified as FDR significant (FDR adjusted p value (q) < 0.05) in our analysis. These EWAS associations were replicated in PIAMA at nominal significance level ($p < 0.05$), but were not significant at the Bonferroni adjusted p value threshold ($p < 2.46x10^{-4}$). Replicated associations for past year $PM_{2.5}$ exposure and differential CpG DNAm are shown in Table 4. The majority of the differentially methylated CpGs that replicated are involved in carcinogenesis pathways (*LASS4, IRX2, AGAP1, MEIS1/2, TNFRSF21, GRIK3, UIMC1*), and several have been specifically implicated in lung cancer (*LASS4, IRX2, MEIS1/2, GRIK3*). *TNFRSF21,* one of the carcinogenesis genes with a replicated CpG site with differential DNAm, is also associated with T cell activation. Overall, average $PM_{2.5}$ exposure in PIAMA was significantly higher as compared to Project Viva. See supplemental Fig. 1 for plot of PIAMA $PM_{2.5}$ exposure levels.

Regional and Pathway Level DNAm Analyses.

Results from differentially methylated regions are shown in Table 5. Although the specific genes identified in the regional analysis were different than those differentially methylated at the individual CpG level (Table 3), the pathways and cellular processes represented were quite similar. We identified differential regional methylation of cell cycle genes (*CDK2AP1, ZC3HC1, MAD1L1*) and genes involved in carcinogenesis (*CDK2AP1, ZC3HC1, MAD1L1, PHLPP1, SFRP2, PLCH1*). Regional analyses also identified differential methylation of *NXN* (nucleoredoxin), which is associated with responses to oxidative stress and *PHLPP1,* a gene involved in modulation of innate immune responses.

Pathway analysis based on differentially methylated CpGs showed pathway enrichment for ABC (ATP binding cassette) transporters, glycosaminoglycan degradation, and mineral absorption pathways (Table 6).

4. Discussion

In this work, we identified associations of exposure to $PM_{2.5}$ air pollution in the previous year with the nasal cell epigenome in over 500 children from Project Viva in eastern Massachusetts. Our study showed three major findings. First, even at the relatively low air pollution exposure levels experienced by our Project Viva participants, we observed differences in nasal cell DNAm with exposure to $PM_{2.5}$. Second, a consistent signal for differential DNAm of cell cycle and innate immune response genes emerged across our site specific CpG and regional DNAm analyses. Third, it was average past year exposure to $PM_{2.5}$, but not the shorter-term exposures (day of, past week, or past month) that was correlated with altered nasal DNAm profiles. This last finding suggests that average exposure levels must be elevated for an extended period of time in order to alter epigenetic profiles in the upper airways.

Our top results included differential DNAm of *RBCK1*, a cell cycle and innate immune signaling gene. *RBCK1*, is a zinc finger protein gene involved in immune dysfunction (Krenn et al., 2018), inflammatory pathways (Tian et al., 2007) and carcinogenesis (Liu et al., 2019). *RBCK1* is a negative regulator of TNF and IL-1 driven inflammation (Taminiau et al., 2016). Higher $PM_{2.5}$ exposure was associated with higher DNAm of a CpG site in the promoter of *RBCK1*, suggesting that the inflammatory dampening effects of this gene may be reduced with higher exposure to air pollution. In addition to its capacity to modulate inflammation, *RBCK1* may also play a role in carcinogenesis (Yu et al., 2019). Similarly, other differentially methylated CpG sites were located within pathways broadly associated with both inflammatory processes and the cell cycle, including two genes from the mTOR signaling pathway in FDR adjusted analyses (*ULK1, RRN3P2*). The potential relevance of mTOR in human airway response to $PM_{2.5}$, identified here in our EWAS study, is recapitulated in data from experimental models. *In vitro* studies of human bronchial epithelial cells show that inhibition of mTOR following $PM_{2.5}$ exposure promotes production of inflammatory cytokines IL-6 and IL-8 (thereby increasing epithelial inflammation), and enhances autophagy/cellular degradation (thereby increasing epithelial cell damage) (Wu et al., 2019).

CpG site specific and regional analysis also identified differential methylation of genes that have been linked specifically to cancers of the airways. Previous epidemiological studies indicate that $PM_{2.5}$ exposure is carcinogenic (Harrison et al., 2004), and in vitro studies suggest that $PM_{2.5}$ may promote carcinogenesis through epigenetic regulation of the tumor suppressor gene p53 (Zhou et al., 2016). Our results did not show alteration of p53 DNA methylation, although we did identify $PM_{2.5}$ associated changes in methylation of of *CDK2AP1* (associated with lung and nasopharyngeal carcinomas), (Sun et al., 2013; Wu et al., 2019) *SFRP2* (modulator of lung cancer cell apoptosis) (Li et al., 2019), and *PLCH1* (associated with non-small cell lung cancer risk) (Zhang et al., 2013). Furthermore, the majority of the CpGs replicated in an external cohort were found in cancer/cell cycle pathways (*MEIS1, IRX2, GRIK3, AGAP1, U1MC1)* reinforcing the global pattern we observed for epigenetic modifications associated with exposure to $PM_{2.5}$ air pollution. Differential DNAm of *MEIS1, IRX2*, and *GRIK3* are known epigenetic biomarkers of lung cancer (however, it is worth noting that *MEIS1* and *IRX2* were hyper rather than hypo-methylated as is observed in cancer studies; *GRIK3* is hypomethylated in our study as well as in carcinogenesis) (Pradhan et al., 2013; Rauch et al., 2012). *AGAP1* controls cancer cell invasion, (Tsutsumi et al., 2020) and *U1MC1* is involved in recognition and repair of DNA lesions (Hamdi et al., 2019). However, it is important to note that, given the overlaps between inflammatory pathways and those in carcinogenesis (Greten and Grivennikov, 2019), the pollution-related differential methylation of genes associated with cancer in the airways in our study may simply reflect alterations in inflammatory processes, including pathways involved in tissue homeostasis and repair that do not necessarily give rise to carcinogenesis.

Findings from our pathway analysis did not highlight either immune or cell cycle/cancer pathways, but instead identified enrichment of ABC (ATP binding cassette) transporters. *In vitro* studies of human bronchial epithelial cells exposed to air pollutant particles show altered ABC transporter gene expression, suggesting that the detoxifying action of these proteins may be compromised with exposure to air pollution, with potential implications for airway epithelial barrier integrity (Le Vée et al., 2019).

As far as we are aware, our study is the first large-scale analysis of air pollution exposures and the nasal cell DNA methylation. One previous study by Somineini et al, (Somineni et al., 2016) focused on the association between TRAP (traffic associated air pollutant) exposure and nasal DNAm (assessed using a 450 K array) in a very small group of 12 African American children with asthma and their non-asthmatic siblings. That report identified lower DNAm of *TET1* in the nasal brushings of participants exposed to higher levels of TRAP; however the investigators did not have the statistical power to look at epigenome-wide associations. In contrast, we did not identify differential DNAm of *TET1* or any other genes associated with global DNAm (i.e. DNA methyl-transferases) in our analyses.

Other studies of air pollution exposure and DNAm have examined circulating blood leukocytes. A comparison of our nasal EWAS findings to studies of $PM_{2.5}$ and blood cell DNAm reveal some genes and pathways related to those identified in our nasal analysis. For example, a previous study in whole blood identified differential methylation of CpGs

within the *NXN* gene (Panni et al., 2016). Our analysis also demonstrated differenial DNA methylation of *NXN* in our regional (but not CpG site specific) findings. *NXN* encodes for nucleoredoxin, a redox dependent regulator which, when activated, may promote Wnt-mediated cell growth and differentiation under conditions of oxidative stress. It is conceivable that air pollutant exposures trigger a cellular turnover process mediated through oxidative stress response genes such as *NXN*. Other reports on $PM_{2.5}$ exposure and blood DNA methylation show differences in immune signaling genes. For example, in candidate gene studies, $PM_{2.5}$ exposure was associated with hypermethylation of the IL-6 gene in circulating leukocytes (Bind et al., 2014). In our nasal EWAS we observed differential DNAm of genes within the mTOR pathway, which is known to modulate IL-6 levels, but we did not observe differential DNAm of the IL-6 gene itself. (This could be because nasal swab samples are enriched for epithelial cells (upstream effector cells) whereas the blood tissue compartment has a higher proportion of cells (i.e. monocytes) that are major producers of IL-6). Other candidate gene epigenetic studies have found associations with differential DNAm of Foxp3 and IL-10, which suggest alterations in adaptive immune response (specifically T-cell responses) with $PM_{2.5}$ exposure (Prunicki et al., 2018). In our nasal EWAS, altered DNAm patterns with higher $PM_{2.5}$ exposure levels suggests shifts in innate, rather than adaptive, immune responses in the local environment of the airway. This is perhaps unsurprising, given that the target tissue sampled in our nasal swabs (the respiratory epithelium) plays a major role in modulating innate immune response to inhaled agents (Diamond et al., 2000).

Greater $PM_{2.5}$ exposure was associated with a far greater number of differentially methylated CpGs in our nasal samples as compared to other EWAS studies that examined differentially methylated CpGs in blood. The largest EWAS of $PM_{2.5}$ exposure to date (>8,000 participants) (Gondalia et al., 2019) quantified site-specific DNAm of blood leukocytes on the 450 K array, and identified only one differentially methylated CpG in the CFTR gene (which did not overlap with our nasal EWAS). A *meta*-analysis of $PM_{2.5}$ exposure and the blood DNAm in 9 birth cohort studies identified 14 differentially methylated CpGs (none of which replicated in our cohort) (Gruzieva et al., 2019). Overall, our results suggest that nasal cells are more sensitive to $PM_{2.5}$ induced modifications in DNAm as compared to blood leukocytes, with some overlap observed for the two tissue types. The larger number of differentially methylated sites in our nasal EWAS as compared to blood EWAS is perhaps expected, given the direct contact of nose with airborne particulates, including $PM_{2.5}$.

Epigenetic changes associated with $PM_{2.5}$ exposure in our nasal study and in blood EWAS tend to be relatively small, and additional studies are necessary to understand the functional impact of these changes. However, it is important to note that persistence and reproducibility of similar small scale changes have been observed in response to other types of exposures (particularly maternal smoking in pregnancy), (Breton et al., 2017) suggesting that effect changes of this magnitude may indeed have biological relevance.

Given that $PM_{2.5}$ exposure is associated with both asthma incidence and severity (Guarnieri and Balmes, 2014) it is reasonable to hypothesize that some nasal DNAm signatures in response to $PM_{2.5}$ may overlap with differential DNAm patterns associated with asthma.

Interestingly, when we compared differentially methylated CpGs associated with $PM_{2.5}$ exposure to those we previously found to be associated with current asthma in our Project Viva participants, (Cardenas et al., 2019) we found no overlap for Bonferroni adjusted EWAS hits. While $PM_{2.5}$ associated differential DNAm patterns were contained mainly in pathways associated with the cell cycle and DNA damage/repair mechanisms, asthma associated differential DNAm included multiple genes involved in Th2 response, T cell activation, and mucin production. Examination of beta coefficients in $PM_{2.5}$ nasal EWAS after removing individuals with current asthma from the analysis showed minimal change, suggesting that current asthma status is not a meaningful effect modifier of epigenetic responses to $PM_{2.5}$ exposure.

Our study has several strengths. First, we had a large sample size. Second, we examined differential DNAm in the nasal cells, which is a first point of contact for air pollution exposures, and may also serve as a surrogate for responses in the lower airways. Lastly, we were able to assess exposure to $PM_{2.5}$ with a spatial resolution of 1 1 km with time specific resolution. We should also acknowledge some limitations. We may have had more exposure misclassification of short term $PM_{2.5}$ relative to long-term average exposures, which could potentially explain why most differential DNAm was observed with past year, rather than past week or past day exposures. While outdoor $PM_{2.5}$ exposures are known to correlate with indoor $PM_{2.5}$ (both in terms of composition and concentration), (Liu and Zhang, 2019) we did not have direct assessment of indoor $PM_{2.5}$ exposure levels. We did not have complementary gene expression data, in order to determine whether methylation changes were associated with changes in gene expression levels. Our replication analysis may also have been hindered by differences in DNA methylation assays (450 K vs. 850 K array), differences in $PM_{2.5}$ exposure levels (which were higher in the Netherlands), and differences in nasal sampling procedures and extracted cells (anterior nares vs. inferior turbinate cells). (Cells collected from the anterior nares have very similar methylation patterns and gene expression profiles as those collected from the inferior turbinate, although the proportion of respiratory epithelial cells in anterior nares samples tend to be lower (65% vs. 99%)) (Lai et al). Even given these differences across cohorts, we were still able to detect replication of multiple sites.

Future studies focused on the consistency of CpG assessment between the 450 K and 850 K platforms, specifically utilizing nasal tissue samples, will help inform replication efforts that compare findings across arrays. Specifically, findings from EPIC and 450 K arrays might differ due enrichment of regulatory elements in the EPIC array. Development of an ideal cell type reference panel for epigenetic studies of the upper airways would also reduce bias in nasal epigenomic studies. Lastly, new studies ought to consider potential effect modifiers of air pollutant exposures and nasal epigenome associations. As highlighted in a recent review, physical activity and diet (particularly B vitamins) may modulate the effects $PM_{2.5}$ on DNA methylation (Rider and Carlsten, 2019). In conclusion, we report multiple associations of long term (past year) $PM_{2.5}$ exposure and the nasal methylome, that were not observed for shorter term exposure windows. Site specific and regional DNA methylation changes were mainly observed in cell cycle, cancer and immune/inflammatory pathways. Our results suggest that the nasal methylome is sensitive to long-term ambient $PM_{2.5}$ exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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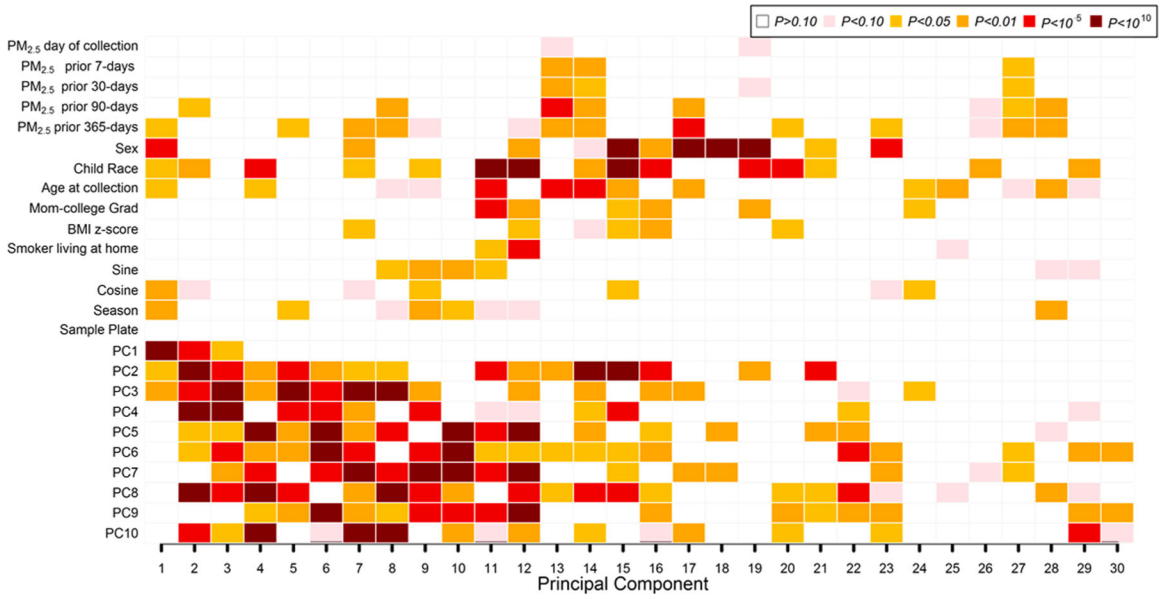

Fig. 1.

Associations of $PM_{2.5}$ exposures, participant characteristics, and cell type heterogeneity with Global Nasal DNA methylation (DNAm) variability. PCs 1–10 on the vertical axis reflect DNAm differences in cell types estimated using the bio-informatic method ReFACTor. Univariate regression analysis (with global DNA methylation principal components as outcomes) was performed. P values for univariate associations between all covariates of interest and the top 30 global DNA methylation PCs (shown on the horizontal axis) are color-coded by smallest P value (dark red; P < 10–10) to largest (blank; P > 0.10). In all, global DNA methylation principal components explained 59% of the variance of the nasal DNA methylation data in the x-axis.

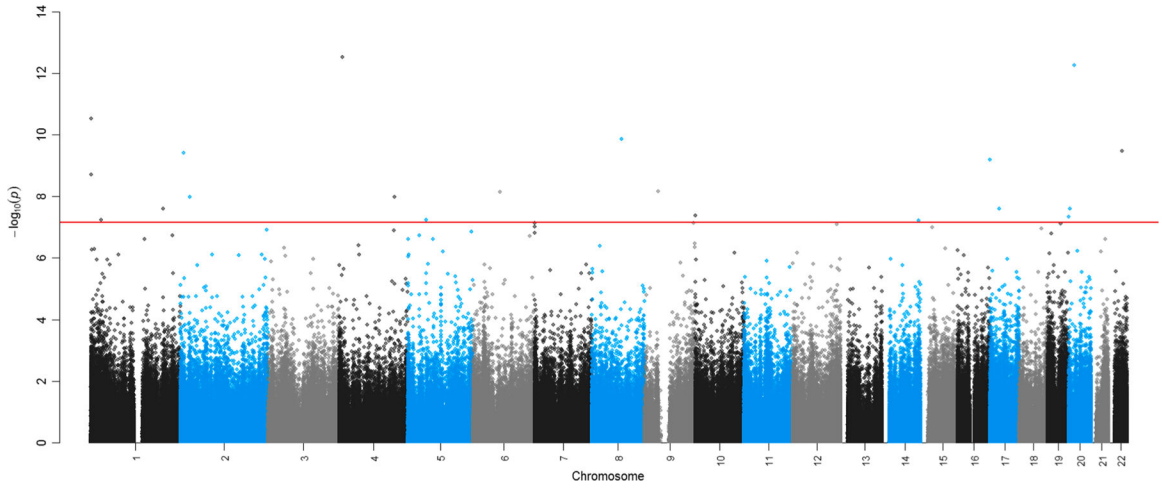

Fig. 2.

Nasal epigenome-wide associations for residence specific past year (365-day) PM2.5 exposure. Y axis shows uncorrected – log10(P values) plotted for each CpG site sorted by chromosomal and genomic position (as shown on the x-axis). Adjustment for multiple testing was accounted for in the epigenome-wide association analyses. Bonferroni threshold for statistical significance ($P < 6.95 \times 10^{-8}$) shown in the solid red horizontal line.

Table 1

Characteristics of Project Viva Adolescents with $PM_{2.5}$ Exposure and Nasal DNA Methylation Data.

Characteristic (N = 503)	N (%) or mean (SD)
Sex	
Female	252 (50.1%)
Male	251 (49.9%)
Age at sample collection (*years*)	12.9 (0.65)
BMI z-score	0.39 (1.06)
Maternal Education	
College graduate	357 (71.0%)
Non-college graduate	146 (29.0%)
Smoker Living in Household	
Yes	59 (11.8%)
No	444 (88.2%)
Current Asthma *	
Cases	59 (13.6%)
Controls	375 (86.4%)
Race/Ethnicity	
White	345 (68.6%)
Black	79 (15.7%)
Hispanic	18 (3.6%)
Asian	15 (3.0%)
More than one race	46 (9.1%)
Season of Sample Collection	
Summer	166 (33.0%)
Fall	108 (21.5%)
Winter	96 (19.0%)
Spring	133 (26.4%)
$PM_{2.5}$ ($\mu g/m^3$)	
Day of sample collection	7.44 (3.05)
7-day average	7.42 (2.00)
30-day average	7.44 (1.53)
90-day average	7.50 (1.09)
365-day average	7.76 (0.55)

* Participants with past asthma and no current wheeze/asthma medication use or current wheeze without asthma not included in N; percentages are out of N = 434

Table 2

Summary of differentially methylated CpGs and DMRs of Nasal EWAS for $PM_{2.5}$ *

	λ	FDR < 0.05	Bonferroni	DMRs
Day of sample collection	0.93	0	0	0
Prior 7-days	0.93	0	0	0
Prior 30-days	1.03	0	0	0
Prior 90-days	1.03	9	2	0
Prior 365-days	1.15	362	20	10

* Bacon-adjusted EWAS results, adjusted for cell type (ReFACTor PCs), child race/ethnicity, sex, BMI *z*-score in early teen, age at nasal swab collection (days), maternal education, smoker living at home, sine and cosine for season at sample collection.

λ = Genomic inflation

FDR: False Discovery Rate (5%)

Bonferroni: $P < 6.953x10^{-8}$

DMR: Differentially Methylated Region (≥2-CpGs and Stouffer $P < 0.05$)

Table 3

Bonferroni Significant Differentially Methylated CpGs associated with a 1 μg/m^3 increase in past year $PM_{2.5}$ Exposure.

CpG	chr	Average % Methylation	Regression Coefficient (M value)	% Diff in DNAm (Beta)	*P* value	Gene/Closest gene	Relation_to_Island
cg26210521	chr4	88.6	−0.256	−1.70	3.08E-13	*SLC2A9*	OpenSea
cg20396870	chr20	66.5	−0.182	−2.74	5.53E-13	*MACROD2*	OpenSea
cg12120973	chr1	87.3	−0.203	−1.44	3.03E-11	*SCNN1D*	OpenSea
cg22118655	chr22	11.1	0.226	1.47	3.29E-10	*ELFN2*	Island
cg01108434	chr8	86.9	−0.254	−1.96	1.42E-10	*PAG1*	OpenSea
cg07769732	chr2	81.8	− 0.169	−1.69	3.92E-10	*KIDINS220*	N_Shore
cg09969776	chr17	93.8	−0.186	−0.72	6.32E-10	*VPS53*	OpenSea
cg13388025	chr1	84.1	−0.162	−1.40	1.98E-09	*ATAD3C*	N_Shelf
cg02180798	chr9	83.6	−0.141	−1.28	6.91E-09	*SHB*	OpenSea
cg07582070	chr6	85.7	−0.140	−1.13	7.33E-09	*IMPG1*	OpenSea
cg18168844	chr5	13.4	0.253	2.60	5.76E-08	*EMB*	N_Shelf
cg08163906	chr14	9.4	0.288	1.76	6.17E-08	*PPP2R5C*	Island
cg13315471	chr2	74.6	−0.121	−1.55	1.02E-08	*TMEM214*	S_Shelf
cg23369179	chr4	74.0	−0.104	−1.34	1.03E-08	*DCHS2*	OpenSea
cg05103574	chr1	90.4	−0.116	−0.67	5.84E-08	*MECR*	N_Shore
cg04351903	chr1	14.4	−0.116	−0.96	2.46E-08	*LGR6*	OpenSea
cg06118847	chr17	78.7	−0.108	−1.21	2.48E-08	*WSB1*	N_Shore
cg01219087	chr20	87.2	−0.152	−1.11	2.53E-08	*PNRP*	Island
cg17608529	chr10	96.9	−0.084	−0.16	4.12E-08	*DIP2C*	Island
cg10495669	chr20	9.0	0.319	1.74	4.69E-08	*RBCK1*	

Table 4

Replication of Project Viva past year $PM_{2.5}$ exposure FDR adjusted results in the PIAMA Cohort.

		Project Viva					PIAMA		
CpG	**Chr**	**Average % Methylation**	**Beta coefficient (Mval)**	**% Diff DNAm (Beta)**	***q* value**	**Unadjusted *P* value**	**% Diff DNAm (Beta)**	**Unadjusted *P* value**	**Gene/Closest Gene**
cg15187788	19	1.9	0.109	0.13%	0.035	1.290E-05	(Beta)	0.003	*LASS4*
cg24105287	2	12.4	−0.295	−2.31%	0.032	9.000E-06	0.44%	0.004	*MEIS1*
cg22986569	5	83.4	−0.258	−3.11%	0.008	7.710E-07	−2.34%	0.005	*IRX2*
cg08805497	2	97.2	−0.082	−0.15%	0.021	4.250E-06	−2.90%	0.006	*AGAP1*
cg17171920	15	93.9	−0.158	−1.04%	0.045	2.350E-05	−0.32%	0.011	*MEIS2*
cg11524642	7	85.9	−0.188	−1.59%	0.002	9.490E-08	−0.92%	0.022	*C7orf50*
cg02643778	6	44.6	0.146	2.56%	0.015	2.140E-06	−0.79%	0.025	*TNFRSF21*
cg04817870	17	85.8	−0.139	−1.08%	0.041	2.020E-05	1.52%	0.033	*ASPSCR1*
cg07680195	19	43.6	0.114	2.00%	0.012	1.710E-06	−0.65%	0.035	*PGLS*
cg05589454	1	89.5	−0.150	−1.12%	0.021	4.240E-06	0.76%	0.045	*GRIK3*
cg19755108	5	49.2	0.150	2.66%	0.003	1.390E-07	−0.51%	0.050	*UIMC1*

Table 5

Differentially Methylated Regions (DMRs) associated with past year $PM_{2.5}$ Exposure.

region	chr	UCSC RefGene	CpGs (N)	Start (bp)	End (bp)	Z statistic	Adjusted *P* value
1	10	*WDFY4*	6	49,892,943	49,893,406	−5.573	0.020
2	12	*CDK2AP1*	3	123,752,805	123,752,916	−6.317	<0.001
3	17	*NXN*	4	800,100	800,717	−5.804	0.005
4	7	*ZC3HC1*	3	129,691,325	129,691,449	5.659	0.012
5	18	*PHLPP1*	4	60,381,593	60,382,247	6.064	0.001
6	7	*MAD1L1*	4	1,991,345	1,991,534	−5.831	0.004
7	11		4	12,088,146	12,088,476	−6.306	<0.001
8	3	*PLCH1*	3	155,422,754	155,423,168	5.510	0.029
9	2	*SPEG*	3	220,355,154	220,355,252	−5.515	0.028
10	4	*SFRP2*	5	154,711,620	154,711,906	−5.470	0.036

Table 6

Biological pathway enrichment analysis of differentially methylated CpGs.

KEGG Biological Pathway	Pathway Name	Genes on Pathway	Differentially methylated genes	Unadjusted *P* value
path:hsa04978	Mineral absorption	58	4	0.0056
path:hsa00531	Glycosaminoglycan degradation	19	2	0.036
path:hsa02010	ABC (ATP Binding Cassette) Transporters	45	3	0.045