Clinical Epigenetics

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Vitamin C supplementation to pregnant smokers alters asthma- and allergy-associated CpGs in child buccal DNA at 5 years of age

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Abstract

Background We previously reported improved respiratory outcomes in babies born to pregnant smokers supplemented with vitamin C (500 mg/day) versus placebo in a randomized clinical trial. Improved respiratory outcomes persisted to 5 years of age and were associated with buccal DNA methylation (DNAm) measured using the InfiniumMethylationEPIC array. The objective of this study was to examine associations of vitamin C treatment and lung function with buccal DNAm using a custom-content Asthma&Allergy array enriched for asthma and allergy loci likely to have a functional impact on gene expression.

Results We profiled DNAm at 36,999 CpGs in loci previously associated with asthma or allergic diseases using custom-content Asthma&Allergy arrays in 137 subjects (65 placebo; 72 vitamin C) with pulmonary function testing (PFT) at the 5-year visit in the "Vitamin C to Decrease the Effects of Smoking in Pregnancy on Infant Lung Function" (VCSIP) double-blind, placebo-controlled randomized clinical trial. We examined the association of buccal DNAm with (1) vitamin C treatment vs placebo, (2) forced expiratory flow between 25 and 75% of expired volume (FEF_{25-75}) and (3) wheeze at 4–6 years of age. We identified 9 genome-wide differentially methylated CpGs (DMCs; FDR < 0.05) and 2 differentially methylated regions (DMRs) between vitamin C and placebo subjects and one CpG associated with FEF_{25-75} at FDR significance. DNAm at 5 CpGs mediated a significant proportion of the vitamin C treatment effect on lung function, including 2 CpGs annotated to the *SLC25A37* gene involved in mitochondrial iron transport.

Conclusions Our study revealed association of in utero vitamin C supplementation and childhood lung function with DNAm at novel loci, providing additional insight toward potential mechanisms for the persistent effects of vitamin C supplementation to pregnant smokers.

Clinical trial registration ClinicalTrials.gov, NCT01723696 (Registered on November 6, 2011) and NCT03203603 (Registered on March 28, 2017).

Keywords Smoking, Vitamin C, DNA methylation, Asthma, Allergy, Nicotine, Lung function, Airway, Wheeze, Mediation

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Background

Epigenetic modifications of the genome are responsible for cell- and tissue-type-specific gene expression and for dynamic changes in gene expression throughout development and the life course [1]. The epigenome, and particularly DNA methylation (DNAm), is responsive to environmental exposures and can provide a molecular record of prenatal exposures [2–5]. Previous studies have also demonstrated that DNAm may mediate the persistent effects of prenatal exposures on later health outcomes and disease susceptibility [6–8].

Maternal smoking during pregnancy (MSDP) is a well-established risk factor for adverse fetal, neonatal and childhood health outcomes [9–13]. In particular, infants exposed to MSDP are more likely to have reduced lung function at birth [14, 15]. Longitudinal studies demonstrate that infants with lower lung function at birth go on to have lower lung function in childhood and adulthood, with greater risk of wheeze, asthma and COPD [14–19]. Despite the adverse health outcomes associated with MSDP, approximately half of pregnant smokers are unable to quit [20, 21].

Therefore, our group has investigated potential interventions to reduce offspring respiratory morbidity due to MSDP exposure. Based on preclinical data in non-human primates, we demonstrated in two randomized clinical trials that vitamin C supplementation to pregnant smokers improves offspring lung function and reduces occurrence of wheeze [22–24]. These effects are persistent to at least 5 years of age and associated with differential methylation in buccal epithelium [25, 26]. We also showed that the majority of differentially methylated CpGs at 5 years appear to originate before birth and persist, with increasing effect size over time [26]. DNAm profiles in buccal epithelium can serve as a proxy of DNAm profiles in airway epithelium [27, 28], where they likely regulate gene expression changes in response to environmental exposure. Thus, these epigenetic signatures may mediate long-term, downstream effects on lung development, including risk for developing asthma, allergic diseases and COPD.

The InfinityMethylation EPIC BeadChip (EPIC array) used in our previous study is the most commonly used platform to profile the human methylome. Recently, a custom-content Asthma&Allergy DNAm array was developed focusing on loci previously associated with asthma or allergy in nasal epithelium which overlap with genetic regulatory elements likely to have a functional impact on gene expression related to respiratory phenotypes [29]. Loci on the array were selected as follows: 1) within differentially methylated regions (DMRs) identified in nasal epithelial cells of asthmatics vs non-asthmatics using whole-genome bisulfite sequencing (WGBS), 2) from 17 previous epigenome-wide association studies (EWAS) of asthma or allergic disease and 3) from previous genome-wide association studies (GWAS). This array was further validated in nasal epithelium from 280 children of diverse ancestry (mean age = 11.1) and 474 children from a predominantly non-Hispanic white cohort (mean age = 6.3). The goal of this study was to use this Asthma&Allergy array to identify novel loci associated with vitamin C supplementation as potential mediators of respiratory outcomes. An overview of our study design is shown in Fig. 1.

Methods

Study design

This study was a follow-up analysis of our multicenter, double-blind, placebo-controlled RCT that demonstrated improved airway function at 3, 12 and 60 months of age in offspring of pregnant smokers randomized to supplemental vitamin C (500 mg/day) versus placebo [53, 54, 58]. We included subjects with acceptable PFTs from in person visits at 5 years (60 months) of age, sufficient quality buccal DNA for methylation analysis and EPIC array data for estimating cell proportions (n = 65 placebo, 72 vitamin C; Fig. 1). The RCT and follow-up study was approved by each site's Institutional Review Board and monitored by an NIH appointed Data Safety Monitoring Board. We obtained written informed consent from all subjects prior to enrollment [23].

Statistical analysis of patient demographics

Patient characteristics are shown in Table 1. Continuous variables were summarized using mean and standard deviation and categorical variables were summarized in percentages after excluding missing values. We tested for differences between groups using the *tableone* package with default settings [30]. We used Pearson's Chi-square test for categorical variables and t-tests for normally distributed continuous variables. Non-normally distributed variables were assessed for group differences using the Kruskal–Wallis rank-sum test.

Respiratory phenotyping

We measured airway function and wheeze in infants and children born to smoking participants in the RCT as described previously [25]. Spirometry was performed at 5 years of age using a model 6800 spirometer (Vitalograph) and adhering to American Thoracic Society (ATS)/European Respiratory Society (ERS) acceptance guidelines. A standardized respiratory questionnaire (RQ) was administered quarterly to the child's parent or primary caretaker and the occurrence of wheeze between the child's fourth and sixth birthday was a secondary outcome of the study. Wheeze was defined as a

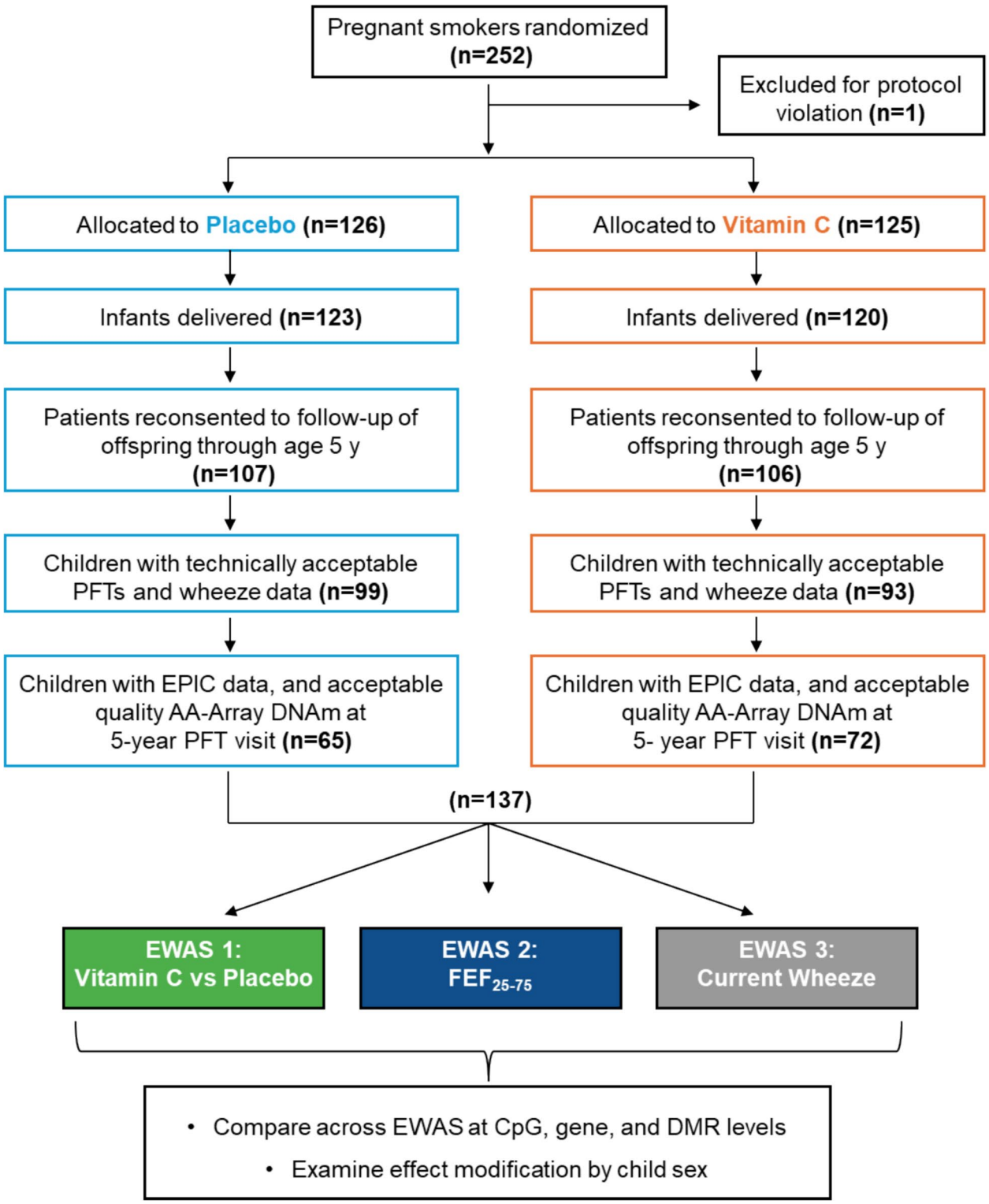

Fig. 1 Study population and design overview

Table 1 Demographics of subjects included in analysis.

	n missing	Placebo (n= 65)	Vitamin C (n=72)	p-value*
Maternal Variables				
Age at enrollment, mean (SD)		27.25 (6.41)	26.61 (5.21)	0.524
Education level, No. (%)				0.188
Less than high school		15 (23.1)	12 (16.7)	
High school degree/ GED		21 (32.3)	36 (50.0)	
Some college		25 (38.5)	22 (30.6)	
Bachelor's degree (BA, BS)		4 (6.2)	2 (2.8)	
BMI at enrollment, mean (SD)		31.06 (8.07)	28.92 (6.74)	0.093
Gravida, mean (SD)		3.68 (2.46)	3.36 (2.24)	0.433
Maternal asthma, No. (%)		23 (35.4)	24 (33.3)	0.942
Study Variables				
Site, No. (%)				0.755
OHSU		13 (20.0)	12 (16.7)	
SWW		33 (50.8)	35 (48.6)	
IA		19 (29.2)	25 (34.7)	
GA at randomization > 18 weeks, No. (%)		32 (49.2)	43 (59.7)	0.289
Plasma Ascorbic Acid (µmol/ L)				
Randomization, median [IQR]	12	46.80 [36.60, 56.40]	47.75 [34.12, 62.90]	0.651
Mid-gestation, median [IQR]	8	37.00 [25.82, 48.30]	56.80 [44.30, 70.60]	**<0.001**
Late-gestation, median [IQR]	21	39.80 [27.70, 47.50]	50.20 [33.95, 68.40]	**0.001**
Smoking Variables				
Maternal Plasma Cotinine (ng/ mL)				
Randomization, median [IQR]	13	55.94 [36.35, 90.56]	68.50 [37.50, 110.66]	0.184
Mid-gestation, median [IQR]	10	56.05 [20.66, 84.96]	54.33 [33.10, 80.92]	0.709
Late-gestation, median [IQR]	24	54.55 [27.30, 86.11]	59.71 [37.40, 83.94]	0.782
Maternal Hair Nicotine (ng/ mg)				
Randomization, median [IQR]	16	2.27 [0.68, 6.27]	3.17 [1.37, 8.92]	0.107
Delivery, median [IQR]	31	1.50 [0.71, 4.64]	2.14 [1.15, 4.57]	0.262
Postnatal 3 months, median [IQR]	12	3.29 [0.90, 6.49]	3.30 [1.17, 8.11]	0.534
Postnatal 12 months, median [IQR]	24	3.91 [1.53, 7.55]	4.37 [1.70, 9.58]	0.351
Postnatal 48 months, median [IQR]	20	1.09 [0.29, 3.50]	2.06 [0.73, 3.85]	0.082
Postnatal 60 months, median [IQR]	12	1.72 [0.39, 4.22]	1.82 [0.69, 3.40]	0.538
Child Hair Nicotine (ng/ mg)				
Delivery, median [IQR]	70	0.33 [0.17, 0.68]	0.27 [0.17, 0.65]	0.78
Postnatal 3 months, median [IQR]	19	2.35 [1.65, 4.71]	3.74 [2.21, 7.00]	**0.035**
Postnatal 12 months, median [IQR]	18	1.55 [0.75, 3.19]	2.09 [1.05, 4.07]	0.097
Postnatal 48 months, median [IQR]	12	0.29 [0.16, 0.91]	0.59 [0.13, 1.46]	0.415
Postnatal 60 months, median [IQR]	7	0.27 [0.08, 0.99]	0.35 [0.13, 0.82]	0.78
Infant Variables				
Gestational age at birth (weeks), mean (SD)		38.50 (1.61)	38.79 (1.83)	0.344
Preterm, No. (%)		3 (4.9)	8 (11.3)	0.317
White, No. (%)		52 (80.0)	54 (75.0)	0.621
Sex = Female, No. (%)		35 (53.8)	32 (44.4)	0.353
Child Variables at 5YO Visit				
PFT height (cm), mean (SD)		113.39 (5.58)	112.52 (4.83)	0.329
FEF_{25-75} (L/ sec), mean (SD)		1.28 (0.31)	1.47 (0.36)	**0.001**
FEF_{50} (L/ sec), mean (SD)		1.43 (0.34)	1.62 (0.37)	**0.003**
FEF_{75} (L/ sec), mean (SD)		0.65 (0.19)	0.79 (0.26)	**<0.001**
FEV_1 (L), mean (SD)		1.14 (0.18)	1.16 (0.19)	0.514
Wheeze at 4-6 yrs of age= Yes, No. (%)		31 (47.7)	21 (29.2)	**0.04**

Table 1 (continued)

*P-values calculated by Chi-square test for categorical data, by Fisher exact test for normally distributed variables and by Wilcox test for non-normally distributed variables.

positive response to any of the following: parental report of wheeze, healthcare professional diagnosis of wheeze or asthma, or any bronchodilator or steroid use. Only children with 1 or more RQs completed after their fourth birthday were included in the analysis.

Collection and processing of DNAm

Methylation profiles in DNA from buccal epithelial cells were measured using the Illumina Allergy&Asthma BeadChip v1.2 following bisulfite conversion at the University of Chicago Genomics Facility [31]. Details of buccal cell collection, DNA extraction and DNAm processing have been published previously [26, 29]. In brief, we removed probes which had either low detection P values, contained a SNP with MAF of at least 5% within 3 bp of the CpG interrogation site or mapped to the X chromosome. Raw probe values were background corrected using preprocessIllumina and quantile normalization using Enmix [32]. The effects of slide, DNA concentration (< or > 12 ng/ml) and extraction batch were removed using ComBat [33]. We used the *EpiDish* R package to estimate proportions of epithelial cells, fibroblasts and total immune cells in our buccal DNA samples [34].

Analysis of differentially methylated CpGs (DMCs) and regions (DMRs)

To assess methylation differences in buccal DNA, we used the logit2 combat-adjusted beta-values (M-values) in linear models adjusted for gestational age at randomization (≤ 18 versus > 18 weeks), clinical site of study, sex, maternal identification as White vs non-White, cell composition and the first latent covariate identified using the *CorrConf* package [35]. In the models for lung function, we added child height at PFT as a continuous variable. Covariates were selected a priori based on RCT design and based on results of surrogate variable deconvolution analysis. Differentially methylated CpGs (DMCs) were defined as having genome-wide significance using the Benjamini–Hochberg procedure (FDR) < 5% and candidate significance using a nominal p-value < 0.001. For each EWAS, we evaluated test statistics inflation and bias using the *BACON* package [36] and quantile–quantile (QQ) plots to visualize expected vs observed p-values.

Differentially methylated regions (DMRs) were assessed using *DMRcate* with recommended settings for array data [37] and the same models used for DMC analysis. DMRs were considered significant if there were 2 or more DMCs within a 1000-bp span and $p < 0.001$ after FDR correction. Consistent with our prior study, we used a second less stringent cutoff criteria to obtain candidate DMRs (pcutoff = 0.1) but required the same level of significance (FDR $p < 0.001$) in filtering the resulting DMRs.

Overlap of treatment and lung function DMCs and mediation analysis

We considered CpGs with a p-value < 0.001 for association with both vitamin C supplementation and $FEF_{25–75}$ as candidates for mediation analysis. For each individual candidate CpG mediator, we fit two linear regression models: (1) the mediator model, with DNAm of the CpG as the outcome and vitamin C treatment as a predictor, adjusting for race, sex, site, GA_strata, PFT height and Epi proportions and (2) the outcome model, with $FEF_{25–75}$ as the outcome and vitamin C treatment as a predictor, adjusting for the mediator, DNAm of the CpG and the covariates from the first model. We used the R package *mediation* [38] with 500 Monte Carlo simulations per CpG to estimate various quantities for causal mediation analysis, including the average direct effects, the average causal mediation effects (ACME; indirect effects) and proportions mediated. We adjusted for multiple comparisons using a false discovery rate (FDR) of 5% (< 0.05) for the ACME tests to determine significant mediation.

Results

Patient demographics

Table 1 summarizes the demographics, cigarette exposure and respiratory outcomes by group. There were no significant differences between the vitamin C and placebo groups for maternal demographics or infant/child demographics. However, there was a tendency for higher cigarette smoke exposure in the vitamin C group based on maternal and child hair nicotine levels. Fasting maternal vitamin C levels were not different between groups at randomization and increased significantly in the vitamin C supplemented group at mid- and late gestation. As previously published, forced expiratory flows were significantly greater, and the occurrence of wheeze at 4–6 years of age was significantly lower, in the vitamin C group versus placebo [25].

EWAS of vitamin C vs placebo

We first examined whether buccal DNAm differed between randomized treatment groups. After adjusting for covariates described in methods, vitamin C treatment was associated with 9 DMCs at FDR-adjusted $p < 0.05$ (5 hypermethylated; 4 hypomethylated) (Fig. 2) and 132

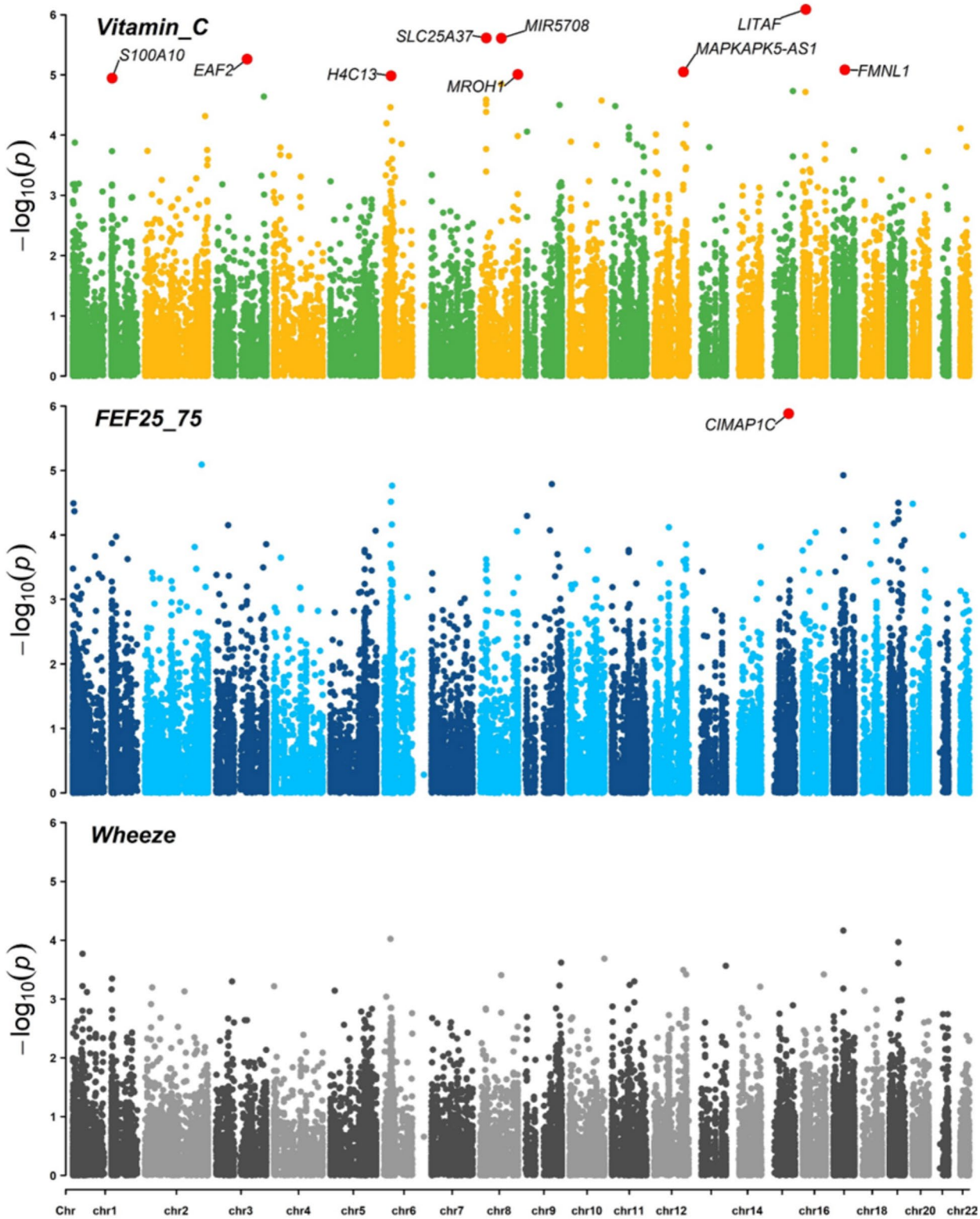

Fig. 2 Manhattan plots depict differentially methylated CpGs associated with vitamin C supplementation (green/yellow), FEF_{25-75} (blue) or wheeze (gray). The chromosomal positions of CpGs are shown on the x-axis. The height of points on the y-axis represents the -log10P-values. Red points labeled with gene symbol indicates FDR significance

DMCs at $p < 0.001$ (57 hypermethylated; 75 hypomethylated; Table S1; Fig. S1), which were considered candidate DMCs for overlap with respiratory DMCs. The top vitamin C treatment DMC was hypermethylated and located upstream of lipopolysaccharide-induced TNF factor (*LITAF*; cg21137660_BC21; log2FC = -0.130). The second most significant DMC was hypomethylated and located within solute carrier family 25 member 37 (*SLC25A37*; cg12309432_BC21; log2FC = -0.284). The third most significant DMC was annotated to microRNA 5708 (cg12309432_BC21; log2FC = 0.246).

We next performed differentially methylated region (DMR) analysis and identified 2 DMRs associated with vitamin C supplementation using a stringent FDR $p < 0.05$ threshold for indexing individual CpGs (Fig. 3). The top vitamin C DMR was annotated to *SLC25A37* and contained 9 CpGs over a 624-bp span (meandiff = -0.030). The second DMR was located in a region overlapping a long noncoding RNA (ENSG00000306274) and upstream of the chemokine (C-X-C motif) receptor 5 (*CXCR5*) gene (3 CpGs; 122-bp span, meandiff = -0.0248). At the less stringent cutoff of 0.1 FDR-adjusted p-value for individual CpGs (with region FDR-adjusted $p < 0.05$), there were 471 candidate DMRs (Table S2).

EWAS of lung function at 5 years of age

We next investigated whether CpGs on the Asthma&Allergy array were associated with lung function measured by spirometry at age 5. One CpG (cg20639654_BC21) annotated to ciliary microtubule-associated protein 1C (*CIMAP1C*) was associated with FEF_{25-75} at FDR significance and 163 CpGs reached candidate significance of $p < 0.001$ (Fig. 2; Fig. S1; Table S3). Using a stringent FDR $p < 0.05$ threshold for individual CpGs, we detected one DMR associated with FEF_{25-75} that spanned 120 bp (6 CpGs; min smoothed fdr = 1.192e-13), located upstream of CCAAT enhancer binding protein gamma (*CEBPG*) and 1,017 candidate DMRs (Table S4). The most significant loci after the *CEBPG* DMR was annotated to *SLC25A37,* spanned 764 bp and contained 11 CpGs (min smoothed fdr = 1.30e-11; mean difference = -0.03).

EWAS of current wheeze at 5 years of age

In our randomized clinical trial of supplemental vitamin C versus placebo to pregnant smokers, we also measured a significant decrease in wheeze in the offspring of pregnant smokers randomized to vitamin C versus placebo (28.3% vs 47.2%; estimated odds ratio, 0.41 [95% CI, 0.23–0.74]; $P = 0.003$) [25]. In this study, the occurrence of wheeze at 4 to 6 years of age was not associated with individual DMCs at FDR significance (Fig. 2; Fig. S1). However, 28 CpGs and 119 DMRs were nominally associated with wheeze at $p < 0.001$ and were used for comparison to other EWAS results (Tables S5 and S6). The most significant candidate DMC associated with wheeze was annotated to migration and invasion enhancer 1 (*MIEN1*), and the top candidate DMR was located in an open sea region upstream of protease, serine, 16 (*PRSS16*) and spanned 949 bp with 25 CpGs hypomethylated in subjects with wheeze (min smoothed fdr = 7.29e-13).

Comparison of vitamin C-associated loci with lung function loci

Out of 132 CpGs associated with vitamin C treatment at $p < 0.001$ and 163 candidate CpGs associated with FEF_{25-75}, 7 overlapped, which is more than expected by chance (hypergeometric $p = 1.4e-07$). Two of the overlapping DMCs were annotated to *SLC25A37*. Three of the 28 candidate wheeze DMCs were also associated with FEF_{25-75} at $p < 0.001$; two were annotated to *CEBPG,* and one to FOS like 2, AP-1 transcription factor subunit (*FOSL2*), which was nominally associated with vitamin C ($p = 0.005$). At the gene level, there were 16 genes containing one or more DMC associated with vitamin C treatment and FEF_{25-75}, 2 genes that overlapped between vitamin C and wheeze DMCs, and 2 genes associated with FEF_{25-75} and wheeze at candidate significance of $p < 0.001$ (Fig. 4A). Out of the 471 candidate treatment DMRs, 129 (27%) intersected DMRs associated with FEF_{25-75} based on genomic coordinates (Table S7). For the 129 regions associated with both vitamin C treatment and FEF_{25-75}, the mean effect sizes were of similar magnitude and direction of association ($r = 0.76$; $p < 2.2e-16$).

Mediation analysis in candidate CpGs

We next tested whether DNAm mediates a significant proportion of the vitamin C treatment effect on lung function in the 7 CpGs which overlapped between vitamin C and FEF_{25-75} DMCs at $p < 0.001$ using causal mediation analysis. We considered mediation to be significant if the FDR-adjusted p-value for the average causal mediation effect (ACME) was less than 0.05. Of the 7 CpGs tested, 5 demonstrated significant mediation (Table S8), including 2 CpGs annotated to *SLC25A37* (Fig. 4B) located within a larger DMR and enriched for the H3K27Ac histone mark based on ENCODE. The CpG with the most significant ACME within *SLC25A37* was cg12309431_TC11 (ACME: 0.0458; 95%CI: 0.010, 0.094; $p = 0.004$) which mediated 19.83% (95%CI: 4.03%, 54.93%) of the total effect of vitamin C on FEF_{25-75}. Three other CpGs demonstrated FDR significant ACME, one annotated to *MAPKAPK5*, one annotated to *USP18* and one to *KIAA2026* (commonly known as *BRD10*), all located in promoter regions (Table S8).

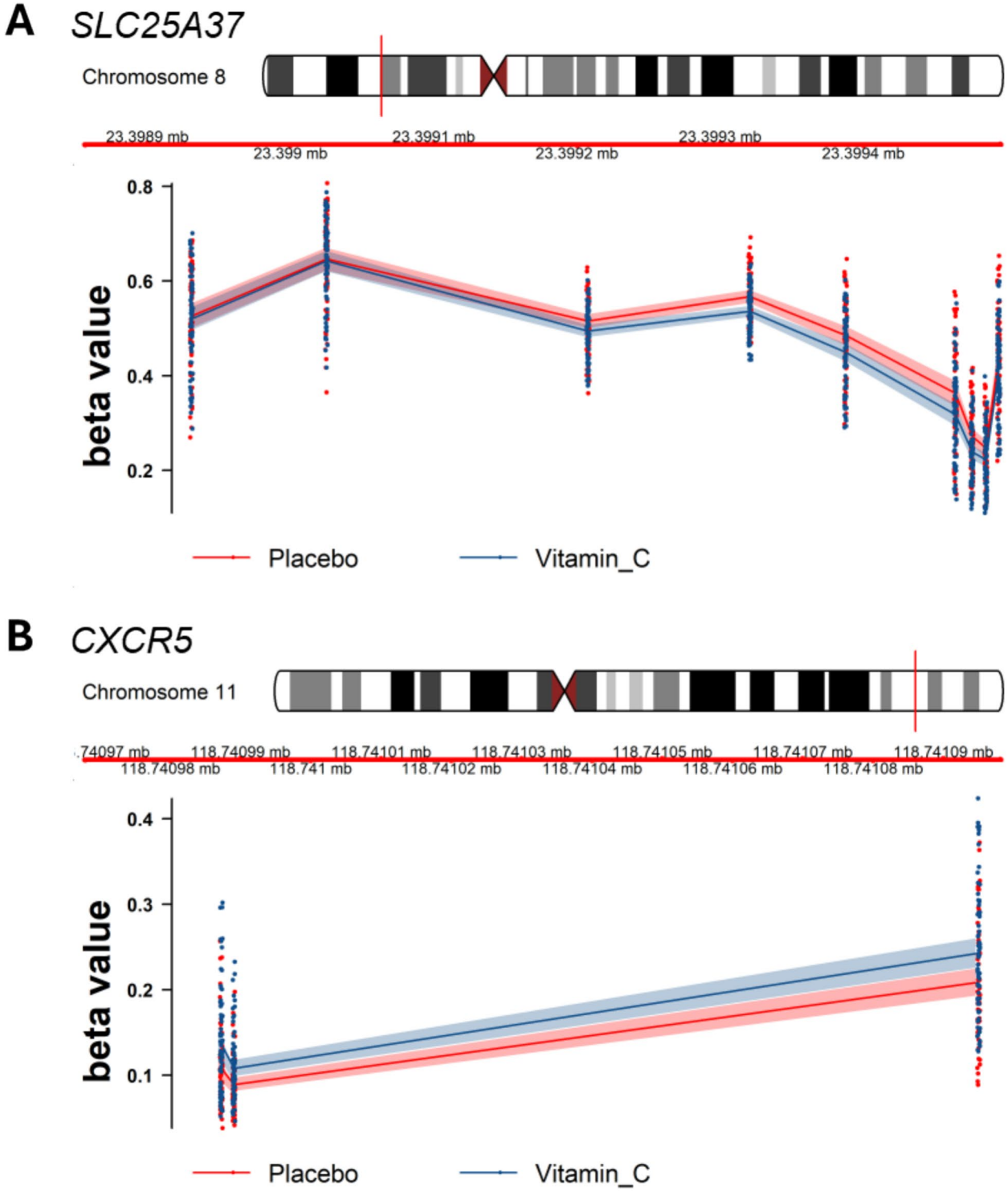

Fig. 3 Gviz plots of significant DMRs between vitamin C supplemented smokers and placebo. Each panel represents a single DMR with upper tracks showing the genomic position and coordinates of the DMR. Each point represents an individual sample for each CpG, colored by treatment group with placebo subjects in red and vitamin C subjects in blue. The y-axes show the methylation values per CpG and sample. The lines and shaded areas represent the mean and 95% confidence intervals for each group. **A** DMR annotated to *SLC25A37*. **B** DMR annotated to *CXCR5*

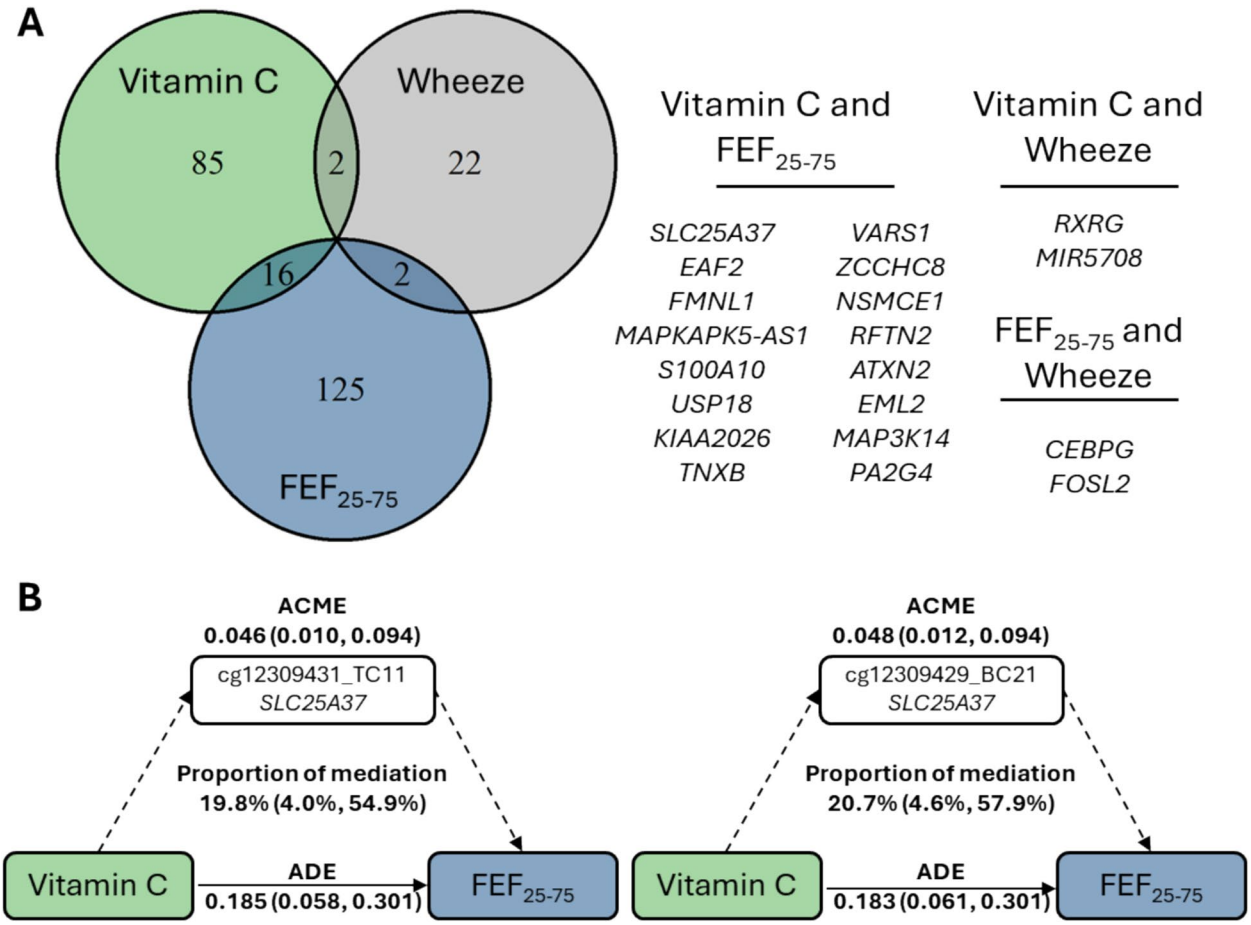

Fig. 4 Overlap between EWAS and mediation analysis. **A** Venn diagram shows the overlap of genes annotated to candidate DMCs ($p < 0.001$) across EWAS with list of genes for each intersection. **B** Mediation diagrams show top CpGs with FDR significant average causal mediation effect (ACME) between vitamin C treatment and lung function (FEF_{25-75}). The mediation models were adjusted for race, sex, site, GA_strata, PFT height and Epi proportions. Results for each path are shown as estimated effect and 95% confidence intervals. Abbreviations: ADE = average direct effects

Vitamin C treatment by sex interaction

Males and females differ in susceptibility to childhood diseases, including respiratory diseases [39]. Therefore, we performed exploratory analyses of sex differences in associations with vitamin C supplementation and lung function. Within the 185 CpGs with an average vitamin C treatment effect $p < 0.001$, the effect sizes in males vs females were highly correlated (R = 0.83; $p < 2.2e\text{-}16$) and none of the overall treatment DMCs had a significant interaction ($p < 0.001$; Table S9), suggesting a similar effect of vitamin C in both sexes. In males alone, there were no FDR significant DMCs between groups and 39 DMCs with nominal $p < 0.001$ (7 were annotated to *SLC25A37*). In females, there were 19 FDR DMCs and 273 candidate DMCs with nominal $p < 0.001$. Out of the 19 FDR DMCs in females, 2 were annotated to *MIR5708*, and 2 were annotated to strawberry notch homolog 2 (*SBNO2;* Fig. S2).

EWAS of lung function by sex

We additionally performed EWAS of lung function stratified by child sex. Similar to the finding of a greater number of vitamin C treatment DMCs in females vs males, we observed 17 CpGs associated with FEF_{25-75} in females at FDR significance and 0 DMCs in males (Fig. S3; Table S10). The effect sizes for the 17 DMCs in females were highly correlated with the overall effect sizes, but larger in magnitude (r = 0.99; $p = 8.6e\text{-}10$). In females, there were 373 candidate DMCs with $p < 0.001$ which mapped to 299 unique genes. The greatest number of candidate DMCs associated with lung function in females were annotated to *MALT1* paracaspase (*MALT1*; 8 CpGs—hypomethylated), followed by *CEBPG* (6 CpGs—hypermethylated).

Discussion

This study profiled DNA methylation in buccal epithelium of 5-year-olds from the VCSIP RCT of vitamin C supplementation in pregnancy [23] using a previously published and validated Asthma&Allergy array, developed using nasal epithelial DNA to cover functional loci associated with asthma and allergic diseases. We additionally tested for association with respiratory outcomes in the same subjects and performed mediation analyses to identify potential biological mechanisms for the observed effect of persistently improved lung function in the vitamin C exposed group. Lastly, we investigated whether associations differ by infant sex as there are known sex differences in prevalence of asthma, with males demonstrating greater susceptibility in childhood [39], and sex differences in lung development and respiratory function [40, 41].

The top DMC in vitamin C compared to placebo was hypomethylated and negatively associated with $FEF_{25-75,}$ located within an active enhancer between a novel lipoprotein amino terminal region containing protein (ENSG00000188897), with enhanced expression in the respiratory system and lymphoid tissues [42] and *LITAF,* which mediates TNF-alpha expression [43]. The Asthma&Allergy array included this locus based on a WGBS DMR in allergic asthmatics vs controls and annotation to active enhancer and transcription factor binding sites [31]. Similarly, multiple EPIC CpGs annotated to *LITAF* were hypermethylated in nasal DNA from atopic children and associated with expired nitric oxide (FeNO) [44, 45].

The most significant DMR associated with vitamin C supplementation was annotated to *SLC25A37* in a region enriched for H3K27Ac, suggestive of a transcriptional enhancer, and was also the most significant DMR negatively associated with FEF_{25-75}. Further, 2 CpGs within this region mediated ~ 20% of the association between vitamin C treatment and $FEF_{25-75.}$ Data from several separate studies have shown hypermethylation in this region associated with FeNO, asthma or atopy in nasal or bronchial epithelial cells [44–46]. *SLC25A37* expression is enhanced in bone marrow and lymphoid tissues [42] and functions as an iron transporter in the mitochondrial membrane. Previous studies have demonstrated a role for mitochondrial iron transporters in the process of ferroptosis, or iron accumulation and lipid peroxidation associated with cell death and fibrosis [47]. Importantly, several recent studies have linked asthma and airway inflammation to ferroptosis-related signaling and suggest inhibitors of ferroptosis as therapeutic targets [48–51]. Cigarette smoke is known to impact iron homeostasis in lung cells [52, 53] and in rats treated with cigarette smoke extract, vascular smooth muscle cell death was prevented by treatment with ferroptosis inhibitors [54]. At physiological (uM) levels similar to the maternal plasma vitamin C levels achieved in this study, vitamin C has been shown to block ferroptosis in vitro [55].

We recently reported that the effect of vitamin C on offspring wheeze was mostly mediated through effects on FEF_{25-75} [56]. Consistent with this, we identified two CpGs positively associated with lung function and negatively associated with wheeze located in the promoter region of *CEBPG* (CCAAT/enhancer binding protein gamma), a transcription factor which also regulates the process of ferroptosis in addition to key antioxidant genes [57, 58]. One additional CpG was associated with both wheeze and FEF_{25-75}, annotated to *FOSL2* (FOS like 2, AP-1 transcription factor subunit; aka *FRA2*), previously shown to be upregulated in several lung diseases, including COPD, pulmonary fibrosis, and both allergic and non-allergic asthma [59]. Therefore, this study suggests that vitamin C may improve offspring lung function and modify prevalence of clinical respiratory symptoms and disease through epigenetic regulation of genes involved in iron homeostasis, ferroptosis and lipid peroxidation [60, 61].

A large meta-analysis of newborn umbilical cord blood and child peripheral blood DNAm identified widespread sex differences, which may contribute to differences in disease susceptibility [62]. Prior studies have also identified sex differences in newborn airway methylation [63], sex-specific peripheral blood DNAm associations with asthma acquisition and lung function [64–66] and sex differences in response to prenatal tobacco exposure in murine lungs [67]. Several plausible mechanisms may explain sex-specific risk of respiratory disease and also associations with DNAm, including documented anatomical, hormonal and genetic differences [40, 68]. For example, sex hormones play an important role in fetal surfactant production and alveolar development, which are thought to explain higher incidence or respiratory distress syndrome in male preterm neonates [41, 69]. In our models with interaction or stratification by sex, we observed a greater number of CpGs associated with vitamin C and with lung function in females versus males. The identified loci may provide insight into the pathogenesis of lower prevalence of asthma diagnoses before puberty among females, although further work in a larger general population is needed.

Few studies have performed EWAS of lung function in childhood specifically, with the majority conducted in blood samples. The strongest evidence for association of early life DNAm with lung function comes from a meta-analysis of cord blood DNAm from 1,688 children (age 7–13) from 5 cohorts participating in the Pregnancy and Childhood Epigenetics (PACE) consortium. The authors

identified several DMRs associated with lung function, with strong signals annotated to genes associated with respiratory development (e.g., *HOXA5, CLCA1, NUDT12 and TCL1A)* [70]. The Isle of Wight Birth Cohort (IOW) has examined peripheral blood DNAm at 10 and 18 years of age and association with lung function cross-sectionally, changes in lung function during adolescence and trajectories of lung function [64, 66, 71, 72]. Results varied by age and sex, and some loci were replicated in external cohorts. Additionally, IOW identified differential methylation of 8 CpGs in blood at birth associated with lung function trajectories (low vs high) with similar direction of effect in a second cohort [73]. An EWAS for lung function using nasal epithelium from 547 children (mean age 12.9 y) participating in Project Viva found no epigenome-wide significant loci [44].

A unique strength of this study is our ability to combine epigenome-wide buccal DNAm analysis with measurements of lung function from the same study visit in preschool children born to pregnant smokers and randomized to an in utero intervention. This is the first EWAS to use this novel Asthma&Allergy array in buccal epithelium. Although DNAm profiles are specific to tissue and cell type, buccal epithelium can serve as a reasonable surrogate for airway epithelium based on conserved methylation profiles and gene expression (27, 28). Additionally, we identified significant correlation between the mean methylation per CpG in buccal DNAm from this study and the mean methylation in published nasal epithelium data (GSE220874; cor = 0.86, $p < 2.2e\text{-}16$).

In this study, we identified 1,017 candidate DMRs associated with lung function which contained 22% of CpGs on the Asthma&Allergy array. In comparison, our previous study using the EPIC array identified 2,277 candidate lung function DMRs, representing 1.63% of CpGs on the EPIC array [26]. Furthermore, the majority (~ 90%) of differentially methylated loci identified in this study were annotated to genes not identified using EPIC, although only a small proportion (1.7%) of genes included in the Asthma&Allergy array are not represented by the EPIC array. These differences are likely due to previously published design differences between platforms, as the Asthma&Allergy array was enriched for known asthma/allergy loci and for transcription factor binding sites (90% of CpGs vs 56% of EPIC CpGs), enhancers (90% compared to 54% of the EPIC array CpGs), introns and exons (24.0% vs 22.1%) and depleted in intergenic regions and 5′ untranslated regions (6.8% vs 7.4%) [29].

We acknowledge several study limitations, including our sample size, which is underpowered compared to large population-based EWAS. As we anticipated that effect sizes for association with vitamin C supplementation and/or respiratory measures would be small relative to effects of prenatal tobacco smoke exposure, we considered a nominal significance threshold of $p < 0.001$ to identify candidates for future validation and comparison with previously published EWAS. We were unable to perform analysis of gene expression in these buccal samples; however, previous work showed significantly more CpGs on the Asthma&Allergy array were correlated with expression of nearby genes in nasal epithelium than CpGs on the EPIC array [29]. As several of the studies used to select loci on the Asthma&Allergy array compared subjects with allergic asthma or allergic sensitization to non-allergic and non-asthmatic subjects [29], we may have missed effects of vitamin C supplementation at loci related to non-allergic respiratory phenotypes. Due to a lack of prior studies examining association of buccal DNAm with lung function in childhood specifically, we were also unable to perform external validation of our results in an independent cohort. Lastly, as this array was developed and characterized in DNA from nasal cells, an important area of future study for this cohort will be to examine DNAm in buccal versus nasal epithelium as our participants age.

Conclusions

This epigenome-wide analysis of buccal DNAm using a recently developed Asthma&Allergy array within a randomized clinical trial population of pregnant smokers receiving vitamin C supplements or placebo identified significant differences at loci previously associated with asthma or allergic disease. In particular, this study identified genes involved in iron homeostasis, ferroptosis and lipid peroxidation. Critically, some of the treatment-associated loci were also associated with lung function measured at 5 years of age and demonstrated significant mediation. Our findings support the potential for DNAm in airway tissues to mediate some of the effects of vitamin C supplementation on lung function and respiratory health in offspring exposed to maternal smoking in utero, as well as suggesting potential mechanisms of action.

Abbreviations

MSDP	Maternal smoking during pregnancy
EWAS	Epigenome-wide association study
EPIC	Illumina Infinium MethylationEPIC BeadChip
Asthma&Allergy array	Custom Illumina Asthma&Allergy array
CpG	Cytosine–guanine dinucleotide
DNAm	DNA methylation
DMC	Differentially methylated CpG
DMR	Differentially methylated region
EWAS	Epigenome-wide association study
FeNO	Fraction expired nitric oxide
RCT	Randomized clinical trial
VCSIP	Vitamin C Supplementation in Pregnancy
IUGR	Intrauterine growth restriction
FDR	False discovery rate
PFT	Pulmonary function test
FEF	Forced expiratory flow
FEF_{25-75}	Forced expiratory flow between 25 and 75% of

	expired volume (also known as MMEF: maximal mid-expiratory flow)
GEO	Gene Expression Omnibus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-01965-2.

Supplementary file1 (XLSX 587 KB)

Supplementary file1 (XLSX 968 KB)

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Author contributions
CTM, RST and ERS initiated, designed and acquired the funding for this study. LESK and ERS coordinated all laboratory analysis. CTM, RST, JH, KM and JB coordinated all clinical data and sample collection. LG and BP provided statistical consultations and LES performed the analysis. CDM and AV oversaw data collection and data integrity. EET and CO provided code and analytical guidance specific to the Asthma&Allergy array. LESK, CTM, RST and ERS drafted the manuscript. All authors contributed to the interpretation of results and revisions to the manuscript. All authors read and approved of the final manuscript.

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Data availability
The raw and processed DNAm used and/or analyzed during the current study are publicly accessible through NCBI Gene Expression Omnibus (GEO) via accession series GSE296777.

Declarations

Ethics approval and consent to participate
The trial was approved by each hospital's institutional review board and monitored by a National Institutes of Health-appointed data safety monitoring board. Written informed consent was obtained from pregnant smokers before enrollment.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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