Vitamin C Prevents Offspring DNA Methylation Changes Associated with Maternal Smoking in Pregnancy

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Abstract

Rationale: Infants whose mothers smoked during pregnancy demonstrate lifelong decreases in pulmonary function. DNA methylation changes associated with maternal smoking during pregnancy have been described in placenta and cord blood at delivery, in fetal lung, and in buccal epithelium and blood during childhood. We demonstrated in a randomized clinical trial (ClinicalTrials.gov identifier, NCT00632476) that vitamin C supplementation to pregnant smokers can lessen the impact of maternal smoking on offspring pulmonary function and decrease the incidence of wheeze at 1 year of age.

Objectives: To determine whether vitamin C supplementation reduces changes in offspring methylation in response to maternal smoking and whether methylation at specific CpGs is also associated with respiratory outcomes.

Methods: Targeted bisulfite sequencing was performed with a subset of placentas, cord blood samples, and buccal samples collected during the NCT00632476 trial followed by independent validation of selected cord blood differentially methylated regions, using bisulfite amplicon sequencing.

Measurements and Main Results: The majority (69.03%) of CpGs with at least 10% methylation difference between placebo and nonsmoker groups were restored (by at least 50%) toward nonsmoker levels with vitamin C treatment. A significant proportion of restored CpGs were associated with phenotypic outcome with greater enrichment among hypomethylated CpGs.

Conclusions: We identified a pattern of normalization in DNA methylation by vitamin C supplementation across multiple loci. The consistency of this pattern across tissues and time suggests a systemic and persistent effect on offspring DNA methylation. Further work is necessary to determine how genome-wide changes in DNA methylation may mediate or reflect persistent effects of maternal smoking on lung function.

Keywords: epigenetics; ascorbic acid; nicotine; prenatal exposure; asthma

At a Glance Commentary

Scientific Knowledge on the Subject: Infants whose mothers smoked during pregnancy demonstrate lifelong decreases in pulmonary function and increased risk of asthma. Exposure to maternal smoking *in utero* is also associated with alterations in DNA methylation at birth, some of which persist into childhood and adolescence.

What This Study Adds to the Field: Vitamin C supplementation administered to pregnant women who will not quit smoking may be a safe and inexpensive measure that improves offspring respiratory function and health through a mechanism involving prevention of alterations in DNA methylation. Identification of methylation targets may point to key pathways underlying the effects of smoking on lung development and the protective effects of vitamin C supplementation.

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Rates of smoking during pregnancy are surprisingly high despite antismoking efforts, and approximately 50% of all smokers will continue to smoke on becoming pregnant (1). It is estimated that at least 12% of infants born in the United States are exposed prenatally to maternal smoking (2). Tobacco smoke exposure *in utero* is the largest preventable cause of low birth weight, preterm delivery, and infant mortality, and is associated with lifelong decreases in pulmonary function and increased risk of childhood asthma (3–7). Thus, understanding the mechanism by which maternal smoking affects lung development and finding ways to prevent those effects are critical.

Epigenetic programming is likely involved in the lifelong consequences of maternal smoking on respiratory function. The epigenome plays a crucial role in the developmental origins of disease, and maternal smoking during pregnancy is now clearly linked to tissue-specific global and gene-specific DNA methylation patterns in the developing fetus, cord blood, and placenta (8–14). Analyses of blood and buccal methylation demonstrate that alterations at some loci persist into childhood and even adolescence (14–16). Moreover, changes in methylation at specific loci are linked to functional consequences of maternal smoking, such as preterm birth, reduced fetal growth, and increased risk of asthma and wheeze (17–20).

Vitamin C (ascorbic acid) is an essential vitamin that cannot be synthesized in humans and needs to be consumed through the diet or dietary supplements to prevent disorders of its deficiency. Cigarette smoking has been shown to significantly reduce levels of circulating vitamin C in the plasma; in pregnant smokers this deficiency extends to the developing fetus (21, 22). In addition to its well-known antioxidant function, vitamin C serves as an essential cofactor to numerous monooxygenases and dioxygenases, including the ten-eleven translocation (TET) enzyme family, which catalyzes the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in the active process of DNA demethylation (23).

In a randomized, double-blind clinical trial (ClinicalTrials.gov identifier, NCT00632476), 500 mg of daily supplemental vitamin C (started at ≤ 22 wk of gestation) administered to women who would not quit smoking during pregnancy improved newborn pulmonary function and decreased incidence of wheeze through

1 year of age (24). We sought to determine whether maternal smoking-associated DNA methylation levels in placentas, cord blood, or buccal epithelia (collected between 3 and 6 yr of age) within this patient cohort were prevented or restored by supplementation with vitamin C in association with improvements in lung function. Some of the results of this study have been previously published in the form of abstracts (25, 26).

Methods

Study Participants and Sample Collection

The recruitment and randomization of participants for the clinical study (Evaluating the Effects of Supplemental Vitamin C on Infant Lung Function in Pregnant Smoking Women; NCT00632476) were conducted in three sites in the Pacific Northwest between March 2007 and January 2011 (24). The study was approved by the institutional review boards at each institution, and written informed consent was obtained for each enrolled patient. Placenta and cord blood samples were collected at the time of delivery from a subset of study participants. Caregivers of infants were later reconsented to conduct follow-up buccal swab sampling in the children.

Targeted Bisulfite Sequencing

We designed a custom pool of biotinylated RNA probes to genes previously identified with methylation and/or expression changes in the offspring of pregnant smokers and additional gene candidates (477 gene regions ± 20 kb; see the online supplement for full targeting strategy) (9, 12, 27–29). Genomic DNA libraries were prepared from placentas ($n = 27$), cord blood ($n = 27$), or buccal swabs ($n = 22$) (ages 3–6 yr), using Methyl-Seq reagents and protocol (Agilent Technologies, Santa Clara, CA). Samples from 41 study participants were used in making libraries. All three tissues were available for 5 subjects, two of three tissues were available for 28 subjects, and one tissue was represented by the remaining 8 subjects (see Figure E1 in the online supplement).

Differential Methylation Analysis

Our bioinformatic pipeline and coverage criteria are described in the online supplement. For each tissue, multiple analysis of variance (ANOVA) for group, sex, and interaction effects was performed,

using a custom R script. Pairwise *post hoc* tests using contrast technique between groups were performed on all CpGs passing the overall significance *F* tests by ANOVA ($P \leq 0.05$), and both unadjusted and false discovery rate (FDR)-adjusted *P* values were retrieved, using the R stats package.

Functional Enrichment Analysis

We used DAVID version 6.7 with default settings for functional annotation clustering (30). The background list included all gene regions captured by our custom probes. We restricted our functional analysis to CpGs associated with maternal smoking and restored by vitamin C. In cord blood and buccal DNAs, our gene lists were limited to FDR-significant CpGs. In placenta we used nominal *P* values, as the FDR cutoff left only one CpG. Results include clusters with enrichment score greater than 1.2 and category Benjamini-Hochberg-adjusted *P* values less than 0.05.

Differentially Methylated Region Analysis

Nominal *P* values for pairwise *t* tests were sorted by chromosome and position, split into chromosome-specific input using R, and read into *comb-p* version 0.32 (31), using the following parameters: dist, 2000; step, 500; seed, 0.05. Differentially methylated regions (DMRs) with a Šidák-corrected *P* value not exceeding 0.05 were annotated to the nearest gene and CpG island, using BEDTools (32) and UCSC Genome Browser tracks for the human genome version hg19 (33).

Validation of DNA Methylation Results

A subset of cord blood DMRs was selected for independent, internal validation (selection criteria described in the online supplement), using bisulfite amplicon sequencing (BSAS). BSAS primers and protocol are provided in the online supplement (Table E8). In addition, external validation by replication analysis was performed by meta-analysis of DNA methylation in newborn cord blood associated with sustained maternal smoking during pregnancy, as described in the online supplement (34).

Respiratory Phenotype Association

We evaluated whether CpGs significantly associated with maternal smoking and restored by vitamin C supplementation were enriched, relative to all CpGs tested, for

Figure 1. DNA methylation changes associated with maternal smoking at targeted loci are blunted by vitamin C supplementation. Heatmaps were generated with CpGs differentially methylated between nonsmokers and placebo groups by pairwise *t* test (nominal $P \leq 0.05$). Raw methylation was $\log_2$ scaled and mean centered for each CpG to normalize distribution before statistical testing (see the *color keys*). Samples from patients born to vitamin C-supplemented smokers (*green*, V1–Vn) cluster closer to the nonsmoking group (*blue*, N1–Nn) than to the placebo group (*red*, P1–Pn) in (A) placenta, (B) cord blood, and (C) buccal epithelium.

Figure 2. Vitamin C treatment reverses the majority of significant DNA methylation changes associated with maternal smoking at targeted loci. Points represent mean methylation for nonsmokers (N), placebo-treated smokers (P), and vitamin C-supplemented smokers (V) at each CpG with significant change in methylation ($\geq 10\%$; nominal $P \leq 0.05$) between the placebo and nonsmoker groups. CpGs are clustered by the direction of change with maternal smoking (HYPER/HYPO) and lines represent the direction of change at each CpG in nonsmokers versus placebo and placebo versus vitamin C mean methylation.

association with either of two phenotypic outcomes measured in our randomized clinical trial: (1) wheeze at 1 year of age and (2) a measurement of newborn pulmonary function: the ratio of time to peak tidal expiratory flow to expiratory time (TPTEF:TE) (24). To test for enrichment, we calculated the hypergeometric probability for hypomethylated and hypermethylated CpGs separately (described in the online supplement).

Results

Patient characteristics of the NCT00632476 cohort were published previously (see Table 1 in Reference 24) along with

data on pulmonary function and wheeze. We achieved greater than $10\times$ sequence depth for 10,902 of the 11,703 probes (93.2%) included in our capture design, which corresponds to more than 70,000 CpGs per tissue (placenta, 79,258; cord blood, 81,461; buccal epithelium, 71,955). On average, the median depth per sample was $45\times$ in placenta, $39\times$ in cord blood, and $37\times$ in buccal epithelium. Covariate analysis (described in the online supplement) identified significant correlation of raw methylation with sex and library batch, prompting us to perform ComBat adjustment for batch effects (35) and to include sex in our statistical model (Figure E2).

In each tissue, we detected more than 3,000 CpGs with variable methylation between groups ($P \leq 0.05$; placenta, 3,112 CpGs; cord blood, 4,527 CpGs; buccal epithelium, 3,378 CpGs). Consistent with previous studies, maternal smoking was associated with significant differences in DNA methylation between the offspring of nonsmokers versus those of placebo-treated smokers (nominal pairwise $P \leq 0.05$; placenta, 1,297 CpGs; cord blood, 2,337 CpGs; buccal epithelium, 1,572 CpGs). Unsupervised hierarchical clustering of CpGs differentially methylated with maternal smoking (based on nominal P value, regardless of magnitude) revealed that DNA methylation of patients born to vitamin C-supplemented

Table 1. Summary of CpG Changes Associated with Maternal Smoking and Reversed by Vitamin C Supplementation

	Placenta	Cord Blood	Buccal Cells	Overall
n (nonsmokers, placebo, vitamin C)	(12, 6, 9)	(11, 8, 9)	(6, 8, 8)	
CpGs tested by ANOVA	79,258	81,464	71,955	
Hypomethylated*	267	300	214	781
Proportion restored (95% CI) [†]	64.42% (58.32–70.10%)	81.33% (76.36–85.49%)	87.85% (82.53–91.77%)	77.34% (74.20–80.20%)
Hypermethylated [‡]	191	209	227	627
Proportion restored (95% CI) [†]	51.31% (44.01–58.56%)	59.33% (52.32–65.99%)	64.32% (57.67–70.47%)	58.69% (54.72–62.56%)

Definition of abbreviations: ANOVA = analysis of variance; CI = confidence interval.

*Mean placebo – mean nonsmoker $\leq -10\%$; nominal $P \leq 0.05$.

[†] – (mean vitamin C – mean placebo)/(mean placebo – mean nonsmoker) $\times 100 \geq 50\%$; proportion and CI calculated with prop.test in R.

[‡]Mean placebo – mean nonsmoker $\geq 10\%$; nominal $P \leq 0.05$.

smokers was more similar to that of nonsmokers than to that of placebo-treated smokers in all three tissues (Figure 1). Remarkably, 77.34% of hypomethylated CpGs and 58.69% of hypermethylated CpGs in placebo versus nonsmokers (nominal pairwise $P \leq 0.05$; change in methylation $\geq 10\%$) were restored by vitamin C treatment by at least 50% of the difference between smokers and placebo (Figure 2 and Table 1).

After removing CpGs affected by sex and performing FDR adjustment of pairwise P values for both nonsmokers versus placebo and placebo versus vitamin C, we identified 203 significantly restored CpGs among all three tissues (top 10 per tissue in Table 2; no magnitude filter). Overlap between tissues at the CpG level was minimal, with only nine CpGs

nominally significant in both cord blood and buccal DNA. In cord blood, 118 restored CpGs (72 hypomethylated with maternal smoking, 46 hypermethylated) mapped to 99 unique loci. Functional annotation clustering revealed enrichment for categories involved in neuronal and placental development, cancer and oncogenesis, chromosomal rearrangement, and drug and glutathione metabolism. In buccal epithelium, 84 restored CpGs (60 hypomethylated, 24 hypermethylated) mapped to 76 unique loci, enriched for gene categories related to signaling and glycoproteins as well as cancer. Only one hypermethylated placental CpG survived FDR multiple testing correction between nonsmokers and placebo, located in a CpG island within the promoter of *ST3GAL1*.

The majority of functionally enriched categories for nominally significant CpGs in placenta included terms related to extracellular matrix (Table E2).

An advantage to targeted bisulfite sequencing is the generation of high-density, single CpG resolution information within a given region, allowing identification of DMRs across contiguous CpGs, which may be more biologically meaningful than methylation rates at individual CpGs. Therefore, we used *comb-p* (31) to identify DMRs between nonsmokers and placebo-treated smokers (referred to as NP DMRs) and between placebo- and vitamin C-treated smokers (PV DMRs). *comb-p* scans across spatially related nominal P values to identify significant regions and applies a distance-related

Table 2. Top Ten CpGs Associated with Maternal Smoking and Restored with Vitamin C per Tissue*

Mapped Gene	Chr	Position (bp)	Nonsmoker (Mean ± SE)	Placebo (Mean ± SE)	Vitamin C (Mean ± SE)	P Value		% Restored†
						Nonsmoker vs. Placebo FDR	Vitamin C vs. Placebo FDR	
Top 10 CpGs in placenta								
ST3GAL1	8	134583256	0.005 ± 0.001	0.092 ± 0.007	0.004 ± 0.001	3.26 × 10 ⁻³	3.12 × 10 ⁻³	101.15
COL11A2	6	33147259	0.936 ± 0.003	1 ± 0	0.9 ± 0.003	6.67 × 10 ⁻²	5.79 × 10 ⁻³	156.25
RXRA	9	137263360	0.95 ± 0.003	0.844 ± 0.006	0.969 ± 0.003	6.67 × 10 ⁻²	1.28 × 10 ⁻²	117.92
NCOR2	12	124893686	0.991 ± 0.001	0.943 ± 0.003	0.994 ± 0.002	6.67 × 10 ⁻²	2.37 × 10 ⁻²	106.25
CCBE1	18	57362673	0.847 ± 0.003	0.773 ± 0.004	0.874 ± 0.005	6.67 × 10 ⁻²	2.47 × 10 ⁻²	136.49
ZCCHC14	16	87442996	0.939 ± 0.003	0.875 ± 0.008	0.965 ± 0.002	6.67 × 10 ⁻²	5.03 × 10 ⁻²	140.63
CCM2	7	45038259	0.627 ± 0.003	0.562 ± 0.012	0.673 ± 0.007	6.67 × 10 ⁻²	5.03 × 10 ⁻²	170.77
CTSK	1	150780799	0.363 ± 0.009	0.204 ± 0.013	0.396 ± 0.01	6.67 × 10 ⁻²	5.17 × 10 ⁻²	120.75
RUNX3	1	25257550	0.112 ± 0.006	0.035 ± 0.007	0.109 ± 0.007	6.67 × 10 ⁻²	5.17 × 10 ⁻²	96.10
RUNX3	1	25277242	0.973 ± 0.003	0.927 ± 0.001	0.984 ± 0.002	6.67 × 10 ⁻²	5.17 × 10 ⁻²	123.91
Top 10 CpGs in cord blood								
HIF1A-AS2	14	62217871	0 ± 0	0.096 ± 0.007	0 ± 0	8.76 × 10 ⁻⁷	1.01 × 10 ⁻⁶	100.00
SOD1	21	33031886	0 ± 0	0.058 ± 0.003	0 ± 0	2.02 × 10 ⁻⁶	1.01 × 10 ⁻⁶	100.00
LIF	22	30642901	0 ± 0	0.045 ± 0.002	0 ± 0	2.49 × 10 ⁻⁴	1.22 × 10 ⁻⁴	100.00
TVP23B	17	18684432	0 ± 0	0.047 ± 0.004	0.004 ± 0.001	3.34 × 10 ⁻³	1.28 × 10 ⁻²	91.49
CNTNAP2	7	145814109	0.038 ± 0.003	0.002 ± 0.001	0.017 ± 0.001	3.34 × 10 ⁻³	4.50 × 10 ⁻²	41.67
MAOB	X	43741933	0.732 ± 0.004	0.474 ± 0.01	0.706 ± 0.009	3.34 × 10 ⁻³	8.18 × 10 ⁻³	89.92
CTNNA2	2	80529735	0 ± 0	0.037 ± 0.001	0.006 ± 0.001	5.12 × 10 ⁻³	2.93 × 10 ⁻²	83.78
CTNNA2	2	80530146	0.048 ± 0.002	0.006 ± 0.001	0.049 ± 0.004	5.12 × 10 ⁻³	1.02 × 10 ⁻²	102.38
COL11A2	6	33116173	0.065 ± 0.004	0.014 ± 0.004	0.047 ± 0.004	8.42 × 10 ⁻³	2.93 × 10 ⁻²	64.71
UBE3C	7	156913122	0.98 ± 0.003	0.882 ± 0.006	0.968 ± 0.003	8.42 × 10 ⁻³	2.83 × 10 ⁻²	87.76
Top 10 CpGs in buccal epithelium								
TTC7B	14	91121520	0.234 ± 0.016	0 ± 0	0.231 ± 0.032	1.46 × 10 ⁻²	1.51 × 10 ⁻²	98.72
ATP10A	15	25955129	0.926 ± 0.011	0.763 ± 0.007	0.936 ± 0.005	2.83 × 10 ⁻²	1.23 × 10 ⁻²	106.13
LOC1019280	1	153518418	0.303 ± 0.014	0.026 ± 0.006	0.296 ± 0.013	3.19 × 10 ⁻²	2.07 × 10 ⁻²	97.47
CATIP	2	219232376	0.058 ± 0.008	0 ± 0	0.053 ± 0.004	3.19 × 10 ⁻²	1.23 × 10 ⁻²	91.38
DLGAP2	8	1501244	0.54 ± 0.02	0.812 ± 0.011	0.647 ± 0.008	3.19 × 10 ⁻²	4.88 × 10 ⁻²	60.66
IGSF21	1	18703322	0.83 ± 0.012	0.956 ± 0.007	0.872 ± 0.006	3.28 × 10 ⁻²	4.39 × 10 ⁻²	66.67
CYP26C1	10	94829036	0.272 ± 0.026	0.062 ± 0.007	0.355 ± 0.027	3.28 × 10 ⁻²	2.15 × 10 ⁻²	139.52
CLSTN3	12	7295553	0.948 ± 0.007	0.762 ± 0.013	0.96 ± 0.004	3.28 × 10 ⁻²	2.15 × 10 ⁻²	106.45
SERPINA3	14	95090393	0.893 ± 0.006	0.757 ± 0.008	0.875 ± 0.01	3.28 × 10 ⁻²	2.81 × 10 ⁻²	86.76
CORO2B	15	68851394	0.101 ± 0.011	0.021 ± 0.005	0.053 ± 0.005	3.28 × 10 ⁻²	3.39 × 10 ⁻²	40.00

Definition of abbreviations: Chr = chromosome; FDR = false discovery rate.

*Top CpGs as ranked by FDR-adjusted P value following pairwise t test between nonsmokers and placebo after removing CpGs with significant sex effects.

†Percent restored calculated as $-(\text{mean V} - \text{mean P})/(\text{mean P} - \text{mean N}) \times 100$, where N = nonsmoker, P = placebo, and V = vitamin C.

correction factor (i.e., P values will be reduced if CpGs nearby are also significant) before applying FDR and multiple-testing correction for each region (described in detail in Reference 31).

The 10 largest DMRs associated with maternal smoking (NP DMRs) and significantly restored by vitamin C (PV DMRs) per tissue are presented in Table 3, and a complete list is provided in Table E3. In cord blood, we identified 43 significantly restored DMRs (Table E4), with the largest located in *RUNX1* (6 CpGs hypomethylated with maternal smoking and restored with vitamin C) (Table 3 and Figure 3A). In placenta, we identified 64 NP DMRs and 108 PV DMRs (Table E5). Among these, 20 DMRs overlapped, and 19 were

restored by at least 50% with vitamin C supplementation (Table E3). The largest significantly restored placental DMR mapped within the promoter of *HIVEP3* (12 CpGs significant after FDR adjustment by *comb-p*; Table 3). Last, in buccal epithelium, 32 of 111 DMRs associated with maternal smoking were significantly restored in the vitamin C-supplemented group versus placebo (Table E6). A DMR mapping to *SLC18A3*, also known as *VACHT* (vesicular acetylcholine transporter), contained the largest number of CpGs restored by vitamin C in buccal DNA (Table E3).

We performed internal technical validation for a subset of 14 cord blood DMRs, using the alternate approach of BSAS. The median depth of coverage for all

321 CpGs covered by BSAS amplicons was $337\times$. Across 14 DMRs (12 genes) we obtained matching targeted bisulfite sequencing (TBS) and BSAS data for 24 patients and 191 CpGs. Correlation analysis demonstrated strong interpatient concordance between TBS and BSAS methylation (Pearson $r^2 = 0.931$; $P < 2.2 \times 10^{-16}$). The difference in methylation between TBS and BSAS for 95.7% of CpGs was within 1.96 SD of the mean difference, showing strong agreement of the two methods and no systematic bias (Figure E3). Both methods identified a pattern of hypermethylation with maternal smoking at *BMP4* and *COL5A1*, and hypomethylation at PR domain-containing 8 (*PRDM8*), *RUNX1*, *NOS3*, *SLC18A3*, *CHAT*, and *HOXA7*.

Table 3. Top Differentially Methylated Regions Associated with Maternal Smoking and Restored with Vitamin C*

Mapped Gene	Chr	Chr Start (bp)	Chr End (bp)	Mean P – N [†]	Mean V – P [‡]	% Restored [§]	# CpGs FDR		Šidák P Value	Absolute Distance to Nearest CpG Island (bp)
							P ≤ 0.05 (comb-p)	P ≤ 0.05 (Pairwise)		
Top 10 DMRs in placenta										
HIVEP3	1	42383975	42385071	−0.091	0.104	114.60	12	12	5.96 × 10 ^{−5}	0
COL4A1	13	110951171	110951346	−0.177	0.147	83.09	3	3	1.53 × 10 ^{−11}	7,546
COL4A2	13	110960293	110961359	0.016	−0.010	62.28	6	3	2.28 × 10 ^{−4}	0
SLC8A1	2	40678493	40678601	−0.056	0.047	83.98	2	2	3.46 × 10 ^{−5}	0
KLHL29	2	23777199	23777252	−0.094	0.095	100.92	2	2	1.54 × 10 ^{−4}	7,798
HOXA4	7	27172606	27172632	0.118	−0.110	92.90	2	2	2.26 × 10 ^{−4}	1,969
CDCA4	14	105499940	105499982	0.093	−0.089	94.87	4	2	2.43 × 10 ^{−4}	0
COL11A2	6	33147254	33147260	0.126	−0.202	159.56	2	2	3.22 × 10 ^{−4}	12,732
DLK1	14	101175833	101175950	−0.134	0.192	142.69	3	2	5.33 × 10 ^{−4}	0
EPHX2	8	27348921	27348932	−0.072	0.077	107.09	2	2	7.68 × 10 ^{−4}	39
Top 10 DMRs in cord blood										
RUNX1	21	36263406	36263794	−0.077	0.100	129.69	7	6	1.21 × 10 ^{−5}	0
HOXA	7	27188195	27188771	−0.107	0.117	109.32	6	5	4.49 × 10 ^{−5}	504
ZSWIM8	10	75545173	75545829	0.024	−0.026	107.38	6	4	1.10 × 10 ^{−10}	0
CYP26A1	10	94834219	94835121	−0.047	0.042	88.69	6	4	1.69 × 10 ^{−7}	0
GJD3	17	38520011	38520082	−0.137	0.164	119.82	4	4	1.21 × 10 ^{−5}	0
PEG10	7	94293859	94294699	0.003	−0.004	152.98	4	4	1.85 × 10 ^{−5}	0
CD14	5	140012294	140013016	−0.050	0.040	81.75	5	4	1.59 × 10 ^{−4}	0
FILIP1L	3	99536309	99536927	−0.028	0.017	59.93	5	3	1.00 × 10 ^{−6}	0
CHAT	10	50821342	50821648	−0.037	0.068	183.48	3	3	1.49 × 10 ^{−6}	0
NUPL1	13	25874931	25875647	−0.055	0.087	158.83	6	3	9.88 × 10 ^{−6}	0
Top 10 DMRs in buccal epithelium										
SLC18A3	10	50819293	50821023	−0.041	0.045	108.99	10	7	6.89 × 10 ^{−6}	0
COL20A1	20	61959687	61960482	0.175	−0.120	68.62	5	5	3.47 × 10 ^{−5}	5,280
EPHB2	1	23110978	23111512	0.053	−0.033	63.27	7	5	5.43 × 10 ^{−5}	0
TFPT	19	54617892	54618399	−0.060	0.061	100.67	4	4	7.35 × 10 ^{−5}	254
HIVEP3	1	42505911	42506606	0.058	−0.044	75.77	6	4	1.62 × 10 ^{−4}	3,932
CD14	5	140011903	140012736	−0.024	0.034	140.87	5	3	1.68 × 10 ^{−7}	0
RXRG	1	165414395	165414605	−0.092	0.047	50.57	3	3	2.90 × 10 ^{−5}	88,068
DLX6	7	96636103	96636289	−0.092	0.082	88.44	3	3	4.04 × 10 ^{−5}	355
HTRA1	10	124222565	124222793	−0.075	0.084	112.45	3	3	1.91 × 10 ^{−4}	326
GNG12	1	68517287	68517365	0.184	−0.142	77.11	4	3	4.50 × 10 ^{−4}	0

Definition of abbreviations: Chr = chromosome; DMRs = differentially methylated regions; FDR = false discovery rate; N = nonsmoker; P = placebo; V = vitamin C.

*Top DMRs as ranked by number of pairwise FDR-significant CpGs in DMR between nonsmokers and placebo.

†Average difference in methylation between placebo and nonsmokers across all FDR-significant CpGs within DMR.

‡Average difference in methylation between vitamin C and placebo across all FDR-significant CpGs within DMR.

§Percent restored calculated as $-(\text{mean V} - \text{mean P})/(\text{mean P} - \text{mean N}) \times 100$.

||The one-step Šidák corrected P value for the DMR region calculated by *comb-p* (31).

Figure 3. Select cord blood differentially methylated regions (DMRs) associated with maternal smoking: (A) Runt related transcription factor 1 (*RUNX1*); (B) growth factor independent 1 transcriptional repressor (*GF11*); (C) aryl-hydrocarbon receptor repressor (*AHRR*); and (D) bone morphogenetic protein 4 (*BMP4*). DMRs were defined with the *comb-p* package to identify enriched regions of spatially related pairwise *P* values between nonsmokers and placebo samples. Plots on the *left* demonstrate the overall trend in methylation changes across the DMR by showing the mean methylation for nonsmokers (N), placebo (P), and vitamin C (V) at each false discovery rate-significant CpG. On the *right*, DMRs are plotted by treatment group with spatial orientation to the genome, with base position on the *x*-axis and methylation (mean $\pm$ SEM) on the *y*-axis.

In both data sets, vitamin C significantly reversed hypomethylation within *PRDM8* (Figure 4 and Figure E4) and *RUNX1* (Figure 3A and Figure E4).

We also examined the generalizability of our findings by correlation with results from a meta-analysis of maternal smoking in newborn cord blood. Meta-analysis by the PACE (Pregnancy and Childhood Epigenetics) Consortium of 13 cohorts identified more than 6,000 CpGs with epigenome-wide association with maternal smoking, including 2,965 CpG loci not previously reaching statistical significance by any individual study (34). Our targeted analysis in cord blood covered 323 of the 6,073 CpGs reaching FDR significance in the meta-analysis (see Table S3 in Reference 34). Of these 323 CpGs, 35 mapped within 24 unique DMRs identified in our study and 26 of 35 CpGs followed a consistent direction of change between meta-analysis data and our results with maternal smoking: six hypomethylated CpGs in *GF11* (Figure 3B), five in the aryl-hydrocarbon receptor repressor gene (*AHRR*) (Figure 3C; one hypo- and four hypermethylated), five hypomethylated in *PRDM8* (Figure 4), two hypermethylated in *BMP4* (Figure 3D) and *ADORA2B*, and two hypomethylated in *CNTNAP2* (Table E7). The coefficients for sustained smoking and newborn methylation by meta-analysis were significantly correlated with mean difference per DMR between nonsmokers and placebo in our study (Spearman $r = 0.5460$; $P = 0.0002$). Among individual CpGs replicated by meta-analysis as significant for association to maternal smoking in cord blood and not in the above-cited DMRs, partial to full reversal of methylation with vitamin C supplementation was identified for CpGs mapping to *RUNX3*, *KLHL29*, *RARB*, *PRDM8*, *AHRR*, *CNTNAP2*, and *FERMT3* (Table 4).

Last, we examined whether methylation changes with maternal smoking at specific loci restored by vitamin C treatment were also associated with respiratory outcomes. We calculated the hypergeometric probability of overlap between restored CpGs and CpGs associated with either incidence of wheeze at 1 year of age or TPTEF:TE at birth. A summary of the results is presented in Table E9. In all three tissues, we observed significant enrichment for restored CpGs associated with pulmonary function at birth (TPTEF:TE; Table E10). We also identified

Figure 4. PR domain-containing 8 (*PRDM8*) is hypomethylated across multiple cord blood differentially methylated regions (DMRs) and restored by vitamin C (VitC). *PRDM8* CpGs identified by meta-analysis as hypomethylated with maternal smoking overlapped with four regions identified in the NCT00632476 cohort with significantly increased methylation in vitamin C-supplemented smokers relative to placebo-treated smokers. The DMR plots (A–D) show the mean $\pm$ SEM methylation for each group in cord blood relative to chromosomal position. (A) chr4_81110074_PRDM8; (B) chr4_81111140_PRDM8; (C) chr4_81118138_PRDM8; (D) chr4_81122567_PRDM8.

significant enrichment for hypomethylated CpGs reversed by vitamin C treatment and associated with wheeze in cord blood. Notably, we identified three or more CpGs in *AHRR*, *RXRA*, *PRDM8*, *DLGAP2*, and *TBC1D16*, which were hypomethylated with maternal smoking and hypomethylated with wheeze, but for which methylation was restored on treatment with vitamin C (full list available in Table E11).

Discussion

It is now well established that maternal smoking during pregnancy causes lifelong

decreases in pulmonary function in offspring and increased risk of asthma (36, 37). Forced expiratory flows in the offspring of smokers are decreased at birth (38), and similar decreases can be measured in high school students (39) and even adults. The mechanism by which *in utero* smoke exposure causes lifelong changes in pulmonary function is unknown, but may involve epigenetics. Consistent with this hypothesis, multiple studies have observed alterations in DNA methylation after *in utero* exposure to maternal smoking (8–10, 13–15, 18, 34, 40).

In agreement with previous reports, our study identified significant changes in DNA

methylation associated with maternal smoking in cord blood and placenta collected at birth, and in buccal epithelium collected between the ages of 3 and 6 years. Maternal supplementation with vitamin C during pregnancy was associated with a reduction or reversal in smoking-related changes across the genome and across tissues at both hypo- and hypermethylated loci (Figure 2). This is in line with a study that found that topical vitamin C restored methylation changes at 88% of hypermethylated regions and 76% of hypomethylated regions in mouse skin treated chronically with a low concentration of benzo[*a*]pyrene, a common carcinogen found in cigarette smoke (41). The direction of change in DNA methylation in response to environmental stressors, such as maternal smoking, is complex and varies according to gene function and proximity to regulatory elements such as promoters and CpG islands (42). However, the underlying mechanism(s) by which maternal smoking/vitamin C affect gene-specific hypo- and hypermethylation have yet to be determined.

RUNX transcription factors play an essential role in lung development, embryonic development, hematopoiesis, and immune responses (43–45). *RUNX1* polymorphisms are associated with airway hyperresponsiveness in pediatric asthma, and expression of *RUNX1* is elevated in embryonic human lung after intrauterine smoke exposure (20). Maternal smoking has been previously associated with hypermethylation of *RUNX3* and *RUNX1* in placenta and cord blood, respectively (9, 13), and with hypomethylation in *RUNX3* at cg27058497 in cord blood (34) (Table 4). In cord blood, we observed *RUNX1* hypomethylation within the proximal promoter that was restored to the level of nonsmokers with vitamin C supplementation, a result we further validated by BSAS (Figure E4). In placenta, we identified two *RUNX3* CpGs among the top 10 CpGs modified with maternal smoking (Table 2) in addition to a hypomethylated CpG 16 bp away from cg24019564 (13). Notably, hypomethylation of *RUNX3* in placenta was restored by vitamin C supplementation, and methylation of *RUNX3* in buccal epithelium was also significantly associated with pulmonary function at birth (Table E10).

We observed widespread hypomethylation of *PRDM8* in cord blood

Table 4. Overlap of CpGs Associated with Sustained Maternal Smoking during Pregnancy in Newborn Blood by Published Meta-analysis with Significant Cord Blood CpGs in the NCT00632476 Cohort

CpG from Meta-analysis	Mapped Gene	Chr	Position (bp)	Meta-analysis Regression Coefficient*	NCT00632476 Cohort	
					Placebo – Nonsmoker	Vitamin C – Placebo
cg27058497	<i>RUNX3</i>	1	25291546	–0.011	–0.078	0.017
cg04535902	<i>GFI1</i>	1	92947332	–0.024	–0.022	–0.073
cg09935388	<i>GFI1</i>	1	92947588	–0.099	–0.037	–0.145
cg25560443	<i>KLHL29</i>	2	23726440	–0.008	–0.115	0.186
cg24230340	<i>HTRA2</i>	2	74758080	–0.009	0.001	–0.073
cg15607292	<i>RARB</i>	3	25426810	0.007	0.045	–0.059
cg27111250	<i>PRDM8</i>	4	81111177	–0.020	–0.115	0.194
cg08606254	<i>AHRR</i>	5	323969	0.025	0.021	–0.128
cg23916896	<i>AHRR</i>	5	368804	–0.012	–0.076	0.026
cg22937882	<i>AHRR</i>	5	405774	0.013	0.053	0.000
cg00976097	<i>AHRR</i>	5	421733	0.012	0.091	–0.049
cg17656608	<i>LOC100505636</i>	5	139486020	0.008	–0.065	0.008
cg16254309	<i>CNTNAP2</i>	7	145814152	–0.003	–0.006	0.024
cg25949550	<i>CNTNAP2</i>	7	145814306	–0.014	–0.014	–0.021
cg11207515	<i>CNTNAP2</i>	7	146904205	–0.023	–0.029	–0.074
cg11694510	<i>IFITM1</i>	11	313354	–0.007	0.106	–0.091
cg20160996	<i>FERMT3</i>	11	63990575	0.002	0.028	–0.078
cg03848267	<i>STAT6</i>	12	57506361	–0.001	0.016	–0.011
cg05923197	<i>BMP4</i>	14	54418804	0.029	0.159	–0.045
cg20244340	<i>SLC24A3</i>	20	19193989	–0.012	0.028	–0.089
cg17072519	<i>AIFM3</i>	22	21322063	0.009	0.168	0.041

Definition of abbreviation: Chr = chromosome.

*Regression coefficient for CpG association with sustained maternal smoking during pregnancy by meta-analysis of 13 cohorts (34).

across five DMRs restored with vitamin C treatment to the level of nonsmokers (Table E4 and Figure 4), an effect also confirmed by BSAS (Figure E4). *PRDM8* encodes a histone methyltransferase belonging to the PR domain zinc finger protein family, whose members have been implicated in malignant transformation of multiple tissues, including lung, through epigenetic silencing mediated by DNA hypermethylation (46). In additional support of our findings, 18 CpGs mapping to *PRDM8* were shown to be significantly hypomethylated with maternal smoking by the meta-analysis (Table E7) (34). Most interestingly, three *PRDM8* CpGs were hypomethylated in cord blood and associated with wheeze at 1 year of age (Table E11).

The CpG most frequently associated with maternal smoking is cg05575921, located in *AHRR* (8–10, 14, 15, 18). *AHRR* has been described as a biomarker for exposure to cigarette smoke (47), and its hypomethylation is also associated with smoking pack-years in both blood and buccal cells of adult smokers (48). We observed hypomethylation at cg05575921 in our cohort that did not reach statistical significance. However, we identified a DMR

61 bp upstream of cg05575921 that was significantly hypomethylated with maternal smoking but not significantly changed with vitamin C (Table E4). Methylation in cord blood at four *AHRR* CpGs outside this DMR were, however, restored by vitamin C by greater than 69% and were also associated with wheeze outcome at 1 year of age (Table E11).

We observed relatively little overlap in DNA methylation patterns between tissues, which is consistent with previous studies of genome-wide differential DNA methylation (9). For example, Novakovic and colleagues identified persistent hypomethylation with exposure to maternal smoking over a region containing cg05575921 in *AHRR* out to 18 months of age in peripheral blood, but no changes in buccal epithelium or placenta (15). Clearly, DNA methylation is highly dynamic in response to environmental insults, in combination with temporal changes that occur during normal differentiation, development, and aging (42).

To further validate our findings we examined replication of our results on the basis of a more recently published meta-analysis (34). Of note, the largest DMR in our data set mapped to *GFI1* (24

hypomethylated CpGs; Table E7), consistent with the meta-analysis and previously associated with maternal plasma cotinine and fetal birth weight (9, 18). Two hypermethylated *BMP4* DMRs in our data set partially overlapped six hypermethylated CpGs in *BMP4* identified by Joubert and colleagues (34). Bone morphogenetic proteins such as that encoded by *BMP4* play a crucial role in lung development, and chronic exposure to cigarette smoke increases expression of *BMP4* in the lungs of Sprague-Dawley rats (49).

Limitations of our study include lack of a cell-type correction model, and therefore it is possible that the observed DNA methylation changes are the result of a shift in the proportions of cell populations (50). However, the top methylation changes identified in other studies of maternal smoking *in utero* are preserved after cell type correction (9, 14). Observed increases in methylation rates among smokers (hypermethylation) could also be indicative of increased 5hmC, which is indistinguishable from 5mC with traditional bisulfite sequencing platforms. For buccal DNA methylation we have not adjusted for postnatal exposure because of

the small sample size. However, it is highly likely that these children continue to be exposed to parental smoking postnatally, given that the women enrolled in this study were unable to quit during pregnancy even with cessation counseling as part of each study visit. Surprisingly, the reversal pattern in DNA methylation levels with vitamin C supplementation persisted in buccal epithelium collected 3–6 years after birth (and the end of vitamin C supplementation), despite likely continued postnatal smoke exposure (Figure 2).

We and others have observed sex-specific DNA methylation differences in response to maternal smoking, and therefore stratified analysis by sex in a larger

cohort may be informative of sex-specific susceptibility to respiratory phenotypes. Samples were collected opportunistically as available and therefore, as described in METHODS, we did not have matching tissues for all samples studied. Because of this, statistical tests were performed in the various tissues separately. Replication with a larger sample size and careful consideration of confounders and covariates are necessary and are currently in progress as part of an ongoing clinical trial, “Vitamin C to Decrease Effects of Smoking in Pregnancy on Infant Lung Function” (NCT01723696).

Last, we focused our initial methylation analysis of this clinical trial cohort on an *a priori* set of genes. Therefore, we were able

to identify enrichment of biological pathways only within these targeted loci. Follow-up studies should include both longitudinal and genome-wide analysis of the methylome to improve our understanding of the molecular targets and potential mechanisms underlying the observed effects of vitamin C on the smoking methylome in association with lung function. If replicated, these findings could have significant implications for the respiratory health of hundreds of thousands of babies born each year who are exposed *in utero* to smoking due to the highly addictive nature of nicotine (2). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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