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DNA Methylation in Babies Born to Nonsmoking Mothers Exposed to Secondhand Smoke during Pregnancy: An Epigenome-Wide Association Study

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Background: Maternal smoking during pregnancy is related to altered DNA methylation in infant umbilical cord blood. The extent to which low levels of smoke exposure among nonsmoking pregnant women relates to offspring DNA methylation is unknown.

Objective: This study sought to evaluate relationships between maternal prenatal plasma cotinine levels and DNA methylation in umbilical cord blood in newborns using the Infinium HumanMethylation 450K BeadChip.

Methods: Participants from the Newborn Epigenetics Study cohort who reported not smoking during pregnancy had verified low levels of cotinine from maternal prenatal plasma (0 ng/mL to <4 ng/mL), and offspring epigenetic data from umbilical cord blood were included in this study ($n = 79$). Multivariable linear regression models were fit to the data, controlling for cell proportions, age, race, education, and parity. Estimates represent changes in response to any 1-ng/mL unit increase in exposure.

Results: Multivariable linear regression models yielded 29,049 CpGs that were differentially methylated in relation to increases in cotinine at a 5% false discovery rate. Top CpGs were within or near genes involved in neuronal functioning (*PRKG1*, *DLGAP2*, *BSG*), carcinogenesis (*FHIT*, *HSPC157*) and inflammation (*AGER*). Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses suggest cotinine was related to methylation of gene pathways controlling neuronal signaling, metabolic regulation, cell signaling and regulation, and cancer. Further, enhancers associated with transcription start sites were enriched in altered CpGs. Using an independent sample from the same study population ($n = 115$), bisulfite pyrosequencing was performed with infant cord blood DNA for two genes within our top 20 hits (*AGER* and *PRKG1*). Results from pyrosequencing replicated epigenome results for *PRKG1* (cg17079497, $\text{estimate} = -1.09$, standard error $(\text{SE}) = 0.45$, $p = 0.018$) but not for AGER (cg09199225; $\text{estimate} = -0.16$, $\text{SE} = 0.21$, $p = 0.44$).

Discussion: Secondhand smoke exposure among nonsmoking women may alter DNA methylation in regions involved in development, carcinogenesis, and neuronal functioning. These novel findings suggest that even low levels of smoke exposure during pregnancy may be sufficient to alter DNA methylation in distinct sites of mixed umbilical cord blood leukocytes in pathways that are known to be altered in cord blood from pregnant active smokers. https://doi.org/10.1289/EHP8099

Introduction

Smoking is consistently linked with alterations in DNA methylation (Philibert et al. 2012, 2013; Wan et al. 2012; Zeilinger et al. 2013). Several epigenome-wide association studies (EWAS) comparing smokers and nonsmokers demonstrate that self-reported smoking is associated with alterations in DNA methylation at multiple cytosine-phosphate-guanine (CpG) dinucleotide sites that can lead to changes in gene transcription (Dogan et al. 2014; Elliott et al. 2014; Shenker et al. 2013; Zaghlool et al. 2015; Zhang et al. 2016; Zhu et al. 2016). There is also evidence to suggest that alterations in DNA methylation can be induced even when people have smoked for only a short time (Philibert et al. 2012, 2013; Prince et al. 2019).

Tobacco smoke exposure *in utero* has also been associated with alterations in DNA methylation across many genes affecting different tissue types (Joubert et al. 2012; Richmond et al. 2015; Suter et al. 2013; Suter and Aagaard 2012). For example, global hypomethylation has been detected in buccal cells and peripheral blood granulocytes of children exposed to prenatal smoking (Breton et al. 2009). Some altered methylated markers have been found to persist over time in the offspring epigenome (Richmond et al. 2015; Wiklund et al. 2019), with studies reporting evidence of persistence across the lifespan from childhood and adolescence (Lee et al. 2015; Richmond et al. 2015) to adulthood (Richmond et al. 2018; Tehranifar et al. 2018).

Smoking-related alterations in DNA methylation have been frequently studied using the umbilical cord blood samples of children exposed to smoke prenatally (Bergens et al. 2019; Ivorra et al. 2015; Miyake et al. 2018; Zhang et al. 2018). The largest meta-analyses of the association between maternal smoking during pregnancy and DNA methylation of newborn umbilical cord blood at over 450,000 CpG sites was conducted by Joubert et al. (2016). Data were meta-analyzed across the Pregnancy And Childhood Epigenetics consortium, which includes data from 13 cohorts ($n = 6,685$). More than 6,000 CpGs were differentially methylated in relation to self-reported maternal smoking, dichotomized as smokers vs. nonsmokers, including 2,965 CpGs corresponding to 2,017 genes not previously related to smoking and methylation in children (Joubert et al. 2016). The top hit was aryl-hydrocarbon receptor repressor (*AHRR*) cg05575921, which has been observed previously as differentially methylated in relation to active smoking in adults and secondhand smoke exposure in children (Joubert et al. 2012, 2016; Monick et al. 2012; Shenker et al. 2013; Zeilinger et al. 2013). Differential DNA

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methylation has also been reported, within Myosin IG (*MY01G*), Growth Factor Independent 1 Transcriptional Repressor (*GFI1*), and *CYP1A1* (Breitling et al. 2011; Joubert et al. 2012; Kirchner et al. 2013; Monick et al. 2012; Shenker et al. 2013). These loci have been implicated in susceptibility to orofacial clefts, tooth development and eruption, asthma, hepatocellular carcinoma, and colorectal and breast cancers (Joubert et al. 2016).

These studies are highly informative to our understanding of the potential consequences of maternal smoking during pregnancy. However, exposure to secondhand smoke during pregnancy among nonsmokers is more common than active smoking during pregnancy. Using data from a U.S. nationally representative study, the Population Assessment of Tobacco and Health Study (2013–2015), our group found that 23% of pregnant women (ages 18–54 y) reported exposure to secondhand smoke, whereas only 6.1% reported smoking during pregnancy (Do et al. 2018). Although the adverse health outcomes associated with secondhand smoke exposure and active smoking during pregnancy are similar for mothers and newborns (Centers for Disease Control and Prevention 2020), the epigenetic consequences on the newborn epigenome of secondhand smoke exposure among nonsmoking women is not known. The reason for this may be the difficulty in assessing secondhand smoke exposure among nonsmoking women. Studies of active smoking during pregnancy have relied on self-report, but assessing secondhand smoke exposure by self-report can be a challenge. There is a risk for bias in self-report measures, especially among pregnant women who are either unaware of their levels of exposure or, because of social desirability, underreport their levels of exposure (Garg et al. 2016; Schechter et al. 2018). A more accurate method to assess secondhand smoke exposure among pregnant women is the use of biomarkers, such as cotinine, a metabolite of nicotine (Philibert et al. 2013).

To our knowledge, no published studies have examined alterations in DNA methylation in infant cord blood as it relates to secondhand smoke exposure during pregnancy. However, there is evidence of associations between secondhand smoke exposure and alterations in DNA methylation in adults from the Multi-Ethnic Study of Atherosclerosis study (Reynolds et al. 2017), as well as experimental evidence of that association in mice (Noël et al. 2017). Knowledge of the DNA methylation loci that may be altered by prenatal secondhand smoke exposure could help identify biomarkers of exposure when maternal cotinine is not available. Equally important to public health is knowing to what extent DNA methylation is altered among nonsmoking women who are exposed to tobacco smoke in their everyday environment. To address these gaps, we conducted an EWAS study to investigate alterations in DNA methylation among a sample of newborns born to nonsmoking pregnant mothers and performed pyrosequencing on select loci in an independent sample from the same cohort to replicate some of our EWAS findings. The results strengthen the case for continued clinical and policy interventions to mitigate any level of smoke exposure during pregnancy, because the findings here appear to suggest that variation, even at lower levels consistent with secondhand smoke exposure, may have the potential to affect the epigenome.

Materials and Methods

Study Population

Participants included in the current analyses are a part of the Newborn Epigenetic STudy (NEST), an ongoing prebirth cohort study designed to improve our understanding of the environmental influences on epigenetic responses and phenotypes in children (Hoyo et al. 2011; Liu et al. 2012). Study participants were identified among pregnant women attending Duke University-affiliated prenatal clinics in Durham, North Carolina, between 2005 and 2011. To be eligible for the NEST study, participants had to be at least 18 years of age or older, English or Spanish speaking, planning to use Duke or Durham Regional Hospital for delivery for the index pregnancy, and willing to provide a prenatal blood sample. Exclusion criteria included women intending to move before the first birthday of the offspring, relinquish custody of the index child, or who had confirmed human immunodeficiency virus (HIV) infection among the first third of the cohort only.

A total of 2,681 mother–child pairs were enrolled and consented. Information on covariates (i.e., race/ethnicity, maternal education, and maternal smoking during pregnancy) was ascertained during the enrollment survey, whereas mother's age at delivery and parity were ascertained through medical records. DNA methylation analyses were completed for 427 with adequate infant umbilical cord blood samples and a minimum amount of follow-up data. Cotinine was assayed from prenatal maternal plasma samples among mother who had singleton births and who had agreed to allow their samples to be used in future research. These cases formed the basis for the analytic samples included in the 450K Beadchip and pyrosequencing analyses, as further described below.

The analytical sample for the 450K Beadchip analyses ($n = 79$) was restricted to those who reported their race/ethnicity as non-Hispanic White or Black and those for whom we had completed cotinine assays from maternal prenatal plasma. In addition, cotinine values had to be less than 4 ng/mL, a threshold proposed by Benowitz et al. as being consistent with secondhand smoke exposure in the U.S. population (Benowitz et al. 2009). Offspring eligibility requirements were limited to live births and singletons. There were no requirements regarding the child's health at birth.

The analytical sample used for validation using pyrosequencing was restricted to those who were not included in 450K Beadchip analyses, those who reported their race/ethnicity as non-Hispanic White or Black, those with cotinine levels lower than 4 ng/mL, and those who had data on necessary covariates for analysis ($n = 115$). Covariates included race/ethnicity (categorical variable, with responses being: Black, non-Hispanic White), mother's age at delivery (continuous variable, reported in years), maternal education [categorical variable, with responses being: less than high school, high school diploma or general education diploma (GED), some college, or college graduate], and parity (categorical variable, with responses being: 0, 1, 2, or 3 or more) for both 450K and pyrosequencing analyses and additional technical covariates (plate, batch) for the 450K Beadchip analyses.

Ethical Approval

The NEST has been approved by the institutional review board at Duke University (Pro00043781, Pro00014548, and Pro00064859) and Virginia Commonwealth University (HM20007857) and have been performed in accordance with the 1964 Helsinki Declaration and its later amendments. Written informed consent was provided by all participants in this study.

Data Collection Methods and Measures

Maternal prenatal cotinine collection and assay procedures. Maternal blood (plasma) specimens were collected with survey data on health, nutrition, stress, and lifestyle behaviors during the enrollment period, which occurred during the first trimester [mean gestational age = 17.3, standard deviation (SD) = 11.3 weeks] for most participants. As described in greater detail elsewhere (Schechter et al. 2018), assays were completed at the Exposure Biology and Chemistry Lab at Duke University. Cotinine was

measured using high-performance liquid chromatography coupled with tandem mass spectrometric detection (HPLC-MS-MS) method. This highly sensitive assay was designed to measure levels of secondhand smoke exposure with a limit of detection (LOD) of 0.05 ng/mL and a reproducibility >94% (Bernert et al. 2009; Dempsey et al. 2012; Jacob et al. 2011). We assumed the value of 0 ng/mL for all values under the LOD for these analyses. No mothers in the 450K Beadchip analyses and 3 mothers in the pyrosequencing analyses had values under LOD.

Assessment of maternal smoking based on self-report and cotinine values. At the time the plasma sample was collected, mothers indicated by survey whether they had smoked at all during pregnancy. Mothers were classified as "nonsmokers" if they had indicated that they had not smoked at any point during their pregnancy. To ensure that our sample included only nonsmoking mothers, we combined the self-reported survey information with cotinine concentration values from prenatal maternal plasma. The resulting analytic sample included only self-reported nonsmoking mothers, who had cotinine concentration levels of <4 ng/mL, with nonzero levels indicative of secondhand smoke exposure according to thresholds proposed by Benowitz et al. (2009). Cotinine concentration values ranging from 0 to 4 ng/mL for maternal plasma were retained as the main exposure of interest for the statistical analyses and was treated as a continuous variable.

Offspring umbilical cord blood collection and DNA methylation. Infant umbilical cord blood was collected via umbilical vein puncture into 10-mL ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tubes, inverted to mix, and centrifuged to harvest plasma and the leukocyte-containing buffy coat used for DNA extraction. Specimens were stored at −80°C until the time of analysis (Murphy et al. 2012). Offspring DNA was extracted using Puregene reagents (Qiagen) according to the manufacturer's protocol. The resulting DNA was assessed for concentration and purity using a Thermo Scientific™ NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific). Offspring genomic DNA (800 ng) was modified by treatment with sodium bisulfite using the Zymo EZ DNA Methylation Kit (Zymo Research). As explained elsewhere (Gonzalez-Nahm et al. 2018), bisulfite treatment of denatured DNA converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged and allowing for quantitative measurement of cytosine methylation status. Methylation levels for individual CpG sites were then measured using the Illumina Infinium® HumanMethylation450 BeadChip (hereafter, "450K Beadchip"; Illumina, Inc.) at the Duke Molecular Genomics Core Facility. The 450K BeadChip interrogates more than 480,000 methylation sites (Bibikova et al. 2011).

Pyrosequencing. We performed bisulfite pyrosequencing using DNA from infant cord blood from a subsample of newborns from the NEST cohort who were not included in 450K BeadChip analyses. Cases were selected from all participants with infant cord blood and prenatal maternal plasma samples not included in 450K analyses and were intentionally selected to be similar to those included in the 450K Beadchip analyses across key maternal characteristics, specifically nonsmoking, Black or non-Hispanic White, and prenatal cotinine levels between 0 to <4 ng/mL. These cases were intentionally selected to be similar to those included in the 450K BeadChip analyses across maternal characteristics.

We assessed DNA methylation at two regions associated with genes within our top 20 hits based on smallest *p*-value demonstrating infant cord blood methylation differences in relation to cotinine concentration from prenatal maternal plasma (*AGER* and *PRKG1*). Pyrosequencing was performed using a PyroMark® Q96 MD pyrosequencer (Qiagen). Assays were designed using the PyroMark Assay Design Software (Qiagen). The Qiagen PyroMark® PCR Kit was used for amplification of the template, using 20 ng of template DNA and 0.12 μL of a 10-μM stock of each forward and reverse primer in a 10-μL reaction volume. Polymerase chain reaction (PCR) conditions were as follows: 95°C × 15 m followed by 60 cycles of 94°C × 30 s, 61°C × 30 s, 72°C × 30 s; a 10-m final extension at 72°C followed by a 4°C hold. PCR primers for *AGER* were F: 5′-biotin-ATA TGT GAT TGG GGG GAT GGT-3′ and R: 5′-CCA CAA AAT AAC CCC AAT AAA CAA-3′ and the sequencing primer: 5′-CCT CCC ACA AAA CCT ATA-3′. The *AGER* sequence to analyze was 5′-CRA AAA CAA AAA AAA TTA AAA ACA CAA C-3′. The underlined CpG position (on the reverse complement strand) corresponds to 450K BeadChip probe cg09199225. For *PRKG1,* PCR primers were F: 5′-biotin-GGA GTT AAA TGG AGA AAG ATA AGG A-3′, R: 5′-CTC TTC CTC AAA ATC CTA CCT AAA T-3′ and the sequencing primer: 5′CTA AAA ACT CTA ATA CTT CA-3′. The *PRKG1* sequence to analyze was 5′-AAT CA ACCT CTC TAA ACA ATT ACA CRC AAA AAA ACC CAC TCT TAA AAA AAT TTC TCC AAA ATC CTT ATC TTT CT-3, with the underlined CpG corresponding to 450K BeadChip probe cg17079497. Assay performance was verified using mixed unmethylated and methylated bisulfite controls (EpiTect DNA; Qiagen). Percent methylation for each CpG was determined using Pyro Q-CpG Software (Qiagen). Linear regression analyses, which included the same variables as covariates in the 450K BeadChip analysis, were performed in SAS (version 9.4; SAS Institute Inc.).

Statistical Analyses

Genome-Wide DNA methylation analysis. To investigate the effect of secondhand smoke exposure among self-reported nonsmoking mothers on newborn DNA methylation, we performed analysis using DNA obtained from infant cord blood on the Infinium® 450K BeadChip. Analysis was performed in R (version 3.4.0; R Development Core Team)/Bioconductor (version 3.5; Bioconductor) environment. Probe intensity data (IDAT) files were processed using the RnBead (version 1.8.0) R package. Probes outside of CpG context, containing SNPs at any probe position, probes for the X and Y chromosomes, and low variability (<0.5%) probes were removed based on best practices recommendations (Chen et al. 2013b; Pidsley et al. 2016). Method wm.dasen was used for data normalization (Pidsley et al. 2016). Background correction was performed with method methylumi.noob (Davis et al. 2020). A matrix of β_{ij} values was obtained. Each β_{ij} value represents the proportion methylated for the ith beadtype on the jth array, which ranges from 0 (unmethylated) to 1 (completely methylated). Quality of each array was assessed by examining density plots of the values, bean plots of all β values, and β values for various control beads. A total of 385,265 probes were left for analyses following quality control procedures. The association between methylation and cotinine level was performed using beta regression as implemented in the betareg (version 3.1-0) R package, which explicitly accounted for the bimodal nature of methylation data (Cribari-Neto and Zeileis 2010). Beta regression has been shown to improve the detection of differential DNA methylation for epigenetic epidemiological studies in comparison to other approaches (Triche et al. 2016). The following covariates were included in the model: race/ethnicity, mother's age at delivery, maternal education, parity, and technical covariates (plate, row, column). Houseman-estimated cell proportions (Houseman et al. 2012) with the Reinius et al. (2012) data set for reference (Reinius et al. 2012) were also included as fixed effects to correct for cell mixture distribution. The resulting *p*-values were corrected using Benjamini and Hochberg's false discovery rate (FDR) method

(Benjamini and Hochberg 1995). CpG sites with FDR <0.05 were considered statistically significant.

Functional enrichment analysis. Functional enrichment analysis was performed using EnrichR (Chen et al. 2013a). The results of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were visualized using the "pathview" R package (version 1.16.7). Given that there were a large number of genes (>11,000) found to be significantly associated with alterations in the DNA methylation of infant cord blood, functional enrichment analyses were run on the top 3,000 genes most highly related to cotinine.

Epigenomic enrichment analysis. As a follow-up to the KEGG functional enrichment analysis, epigenomic enrichment analyses were conducted. Specifically, GenomeRunner (Dozmorov et al. 2012, 2016) was used to test whether hg19 genomic coordinates of CpG sites associated with low-level secondhand smoke exposure (as measured by cotinine levels) were significantly enriched in certain genomic annotations collected by the ENCODE, Roadmap, and other consortia, as compared with randomly sampled CpGs from all CpGs on the 450K BeadChip. Briefly, hypergeometric tests were used to calculate enrichment/depletion at specific CpG sites, and p-values were FDR-corrected for multiple testing. Given that methylation profiles were obtained from blood samples, cell type-specific genome annotation data from Gm12878 cell line (B-cell lymphoblastoma) were used.

DNA methylation analysis at select gene regions by pyrosequencing. Linear regression analyses were conducted to determine associations between CpG markers that were pyrosequenced within two select gene regions and alterations in DNA methylation of umbilical cord blood, including the same covariates in the 450K BeadChip analysis. These analyses were performed in SAS (version 9.4; SAS Institute).

Pyrosequencing assays for *AGER* and *PRKG1* were designed to capture the select CpG sites that were significant for each on the 450K BeadChip platform. CpGs were selected from a larger set of CpGs that were significant in the 450K BeadChip sample, based on statistical significance, the magnitude of effect size, and the ability to design and validate pyrosequencing assays to capture the specific CpG sites represented in the 450K BeadChip platform. Assay design was first validated using defined mixtures of fully methylated and unmethylated bisulfite modified control DNA (0%, 25%, 50%, 75%, and 100% methylated DNA), which showed good agreement between the amount of methylated DNA in the reaction and what was measured by pyrosequencing (*AGER*: $R=0.996$; *PKRG1*: $R=0.996$). Due to the stringent internal quality controls that were imposed for this pyrosequencing reaction, more samples were excluded for *AGER*. We then used these assays to measure DNA methylation in umbilical cord blood for an independent subset of participants in the NEST cohort for whom 450K BeadChip data were not generated.

Results

Sample Characteristics

Sample characteristics and comparisons between individuals included in the 450K BeadChip and pyrosequencing analyses are displayed in Table 1. Of the 79 pregnant women included in the 450K Beadchip analysis, 45.6% ($n=36$) indicated that their race/ethnicity was Black, and 54.4% ($n=43$) indicated that their race/ethnicity was non-Hispanic White. Nearly half ($n=38$, or 48.1%) of the sample were college graduates. For 35.4% ($n=28$) of the sample, the index pregnancy was their first pregnancy. The average age of mothers at delivery was 29.2 y (range: 18–45 y, SD = 6.4 y). Cotinine concentration in plasma from blood samples collected from pregnant women ranged from 0 to <4 ng/mL (mean = 1.0, SD = 0.1; median = 1.0, range: 0.0–3.8 ng/mL). None of the cotinine values were under the LOD. On average, mothers delivered their babies at 39.0 (SD = 1.8; median = 39.1, range: 32.2–41.7) wk gestation.

Of the 115 pregnant women included within the pyrosequencing sample, 65.2% ($n=75$) were Black, and 34.8% ($n=40$) were

Table 1. Sample characteristics.

	Full sample ($n=2,681$)		Included in 450K BeadChip analyses ($n=79$)		Included in pyrosequencing analyses ($n=115$)		
Variable	n (%) or mean ± SD	Median (range)	n (%) or mean ± SD	Median (range)	n (%) or mean ± SD	Median (range)	p-Value
Race/ethnicity							0.01
Black	1,166 (43.6)		36 (45.6)		75 (65.2)		
Hispanic	478 (17.9)		0 (0.0)		0 (0.0)		
Other race/ethnicity	125 (4.7)		0 (0.0)		0 (0.0)		
Non-Hispanic White	908 (33.9)		43 (54.4)		40 (34.8)		
Education status							0.33
Less than high school	526 (21.6)		3 (3.8)		12 (10.6)		
High school/GED	556 (22.9)		19 (24.1)		22 (19.5)		
Some college	542 (22.3)		19 (24.1)		29 (25.7)		
College graduate	807 (33.2)		38 (48.0)		50 (44.3)		
Parity							0.31
0	829 (33.9)		28 (35.4)		48 (41.7)		
1	788 (32.2)		22 (27.9)		35 (30.4)		
2	483 (19.7)		18 (22.8)		23 (20.0)		
3 or more	347 (14.2)		11 (13.9)		9 (7.8)		
Maternal smoking during pregnancy							Not computed
No	1,834 (75.9)		79 (100.0)		115 (100.0)		
Yes	582 (24.1)		0 (0.0)		0 (0.0)		
Mother's age at delivery (y)	28.3 ± 5.9	28.0 (18.0–40.0)	29.2 ± 6.5	30.0 (18.0–45.0)	28.0 ± 5.6	28.0 (18.0–40.0)	0.20
Gestational age at birth (wk)	38.4 ± 2.6	39.1 (18.4–42.3)	39.0 ± 1.8	39.1 (32.2–41.7)	39.2 ± 1.6	39.3 (33.5–41.4)	0.35
Gestational age at maternal plasma collection (wk)	20.1 ± 12.5	13.9 (3.3–42.0)	33.9 ± 11.6	38.9 (3.3–41.6)	13.8 ± 7.8	11.3 (5.3–40.9)	<0.01
Cotinine concentration in maternal plasma (ng/mL)	16.0 ± 47.1	0.6 (0.0–371.0)	1.0 ± 0.7	1.0 (0.0–3.8)	0.7 ± 0.7	0.5 (0.0–3.6)	<0.01

Note. p-Values reflect comparisons between the 450K sample and the pyrosequencing sample only. The p-value for maternal smoking during pregnancy was not computed because both 450K and pyrosequencing analyses included mothers who did not smoke during pregnancy. GED, general education diploma; SD, standard deviation.

non-Hispanic White. Less than half (44.3%, $n = 50$) were college graduates. The average age at delivery was 28.0 y (range: 18 to 40 y; SD = 5.6). Cotinine concentration in plasma from maternal blood samples ranged from 0 to <4 ng/mL (mean = 0.7, SD = 0.7; median = 0.5, range: 0.0–3.6). Three of the cotinine values were under the LOD and assigned a value of 0 ng/mL. Mothers were on average 38.0 (SD = 5.6; median = 39.3, range: 33.5–41.4) years of age when they delivered their babies.

There were no significant differences between those in the 450K BeadChip sample and those in the pyrosequencing sample except for race/ethnicity and cotinine values (Table 1). The percent of Black mothers was greater in the pyrosequencing sample than the 450K BeadChip sample (65.2% vs. 45.6%, $p = 0.01$), and cotinine concentrations were statistically significantly more elevated in the 450K BeadChip analyses relative to those in the pyrosequencing sample (mean = 1.0; SD = 0.7; median = 1.0; range: 0.0–3.8 ng/mL vs. mean = 0.7; SD = 0.7; median = 0.5; range = 0.0–3.6 ng/mL, $p < 0.01$). In addition, the timing of the maternal prenatal plasma collection differed between the 450K BeadChip and pyrosequencing samples (mean weeks of gestational age = 33.9; SD = 11.6; median = 38.9; range: 3.3–41.6 vs. mean = 13.8; SD = 7.8; median = 11.3; range = 5.3–40.9, $p < 0.01$). Histograms of maternal cotinine in plasma (nanograms per milliliter) from 450K BeadChip and pyrosequencing analyses are shown in Figure S1.

Genome-Wide DNA Methylation in Relation to Low-Level Smoke Exposure

Using FDR <0.05 for the overall model fit and adjusting for potential covariates, we found 29,049 CpG sites to be differentially methylated in relation to a 1-unit ng/mL increase in cotinine among nonsmoking mothers (Excel Table S1).

Table 2 summarizes the top 20 CpG sites based on smallest adjusted p-values in relation to cotinine concentration levels among nonsmoking pregnant women in the NEST. The most significant CpG site (cg11407598) is located on chromosome 19 (beta = -0.76, SE = 0.08, adjusted $p = 6.6 \times 10^{-17}$).

The second most significant CpG site (cg09199225) was associated with the *AGER* gene located on chromosome 6 and demonstrated the highest level of hypermethylation found in relation to a 1-unit ng/mL increase in plasma cotinine (beta = 1.38, SE = 0.16, adjusted $p = 1.19 \times 10^{-13}$). In addition to this CpG site, four other CpG sites associated with *AGER* were identified as statistically significant. All five of these CpG sites associated with AGER demonstrated hypermethylation (Excel Table S1). The *AGER* gene lies within the major histocompatibility complex class III region of chromosome 6, which has been implicated in inflammatory and immune responses. It is a highly polymorphic gene that has been associated with the development of lung function, diabetes, and cardiovascular disease (Egaña-Gorroño et al. 2020; Serveaux-Dancer et al. 2019).

The third most significant CpG site (cg05396900) was also hypermethylated (coefficient = 0.44, SE = 0.05, $p = 6.42 \times 10^{-13}$) with a 1-unit ng/mL increase in cotinine levels (Excel Table S1). This CpG site is associated with the *PRKG1* gene, which encodes cyclic GMP-dependent protein kinase 1 and is involved with learning, memory, and circadian rhythm regulation (Langmesser et al. 2009). An additional five CpG sites (two showing hypermethylation and four showing hypomethylation) associated with *PRKG1* showed altered methylation in relation to a 1-unit ng/mL increase in plasma cotinine at FDR-corrected $p < 0.05$ (Excel Table S1).

Table 2. Top 20 CpG sites based on smallest p-value demonstrating cord blood methylation differences in cotinine concentration levels from maternal plasma samples among nonsmoking pregnant women in the newborn epigenetic study ($n = 79$).

CpG Site	Mapped Gene	Chr	beta	SE	Unadjusted p-value	Adjusted p-value	UCSC Referenced Gene Description
cg11407598		19	−0.76	0.08	1.71×10^{-22}	6.59×10^{-17}	
cg09199225	*AGER*	6	1.38	0.16	6.16×10^{-19}	1.19×10^{-13}	Advanced glycosylation end product-specific receptor
cg05396900	*PRKG1*	10	0.44	0.05	5.00×10^{-18}	6.42×10^{-13}	Protein kinase, cGMP-dependent, type I
cg19810457	*MAPK9*	5	−0.63	0.07	1.95×10^{-17}	1.72×10^{-12}	Mitogen-activated protein kinase 9
cg15570148	*FHIT*	3	0.30	0.04	2.23×10^{-17}	1.72×10^{-12}	Fragile histidine triad
cg01588993		12	−0.40	0.05	4.44×10^{-17}	2.85×10^{-12}	
cg01417692	*MOBKL2C*	1	−0.11	0.01	1.44×10^{-16}	7.67×10^{-12}	
cg06432426		2	−0.31	0.04	1.75×10^{-16}	7.67×10^{-12}	
cg06567155	*SLC25A15*	13	−0.51	0.06	1.79×10^{-16}	7.67×10^{-12}	Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15
cg06998640		12	−0.10	0.01	2.23×10^{-16}	8.59×10^{-12}	
cg01373721	*TIMM23*	10	0.13	0.02	8.40×10^{-16}	2.94×10^{-11}	Translocase of inner mitochondrial membrane 23 homolog (yeast)
cg27413290	*ZC3H3*	8	−0.55	0.07	1.86×10^{-15}	5.97×10^{-11}	Zinc finger CCCH-type containing 3
cg14189245	*C11orf10|FEN1*	11	0.46	0.06	2.14×10^{-15}	6.34×10^{-11}	Flap structure-specific endonuclease 1
cg09719342	*STK32A*	5	−0.93	0.12	3.50×10^{-15}	9.64×10^{-11}	Serine/threonine kinase 32A
cg20490386	*CTDP1*	18	−0.11	0.01	3.87×10^{-15}	9.94×10^{-11}	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1
cg14992144	*GHRL|GHRLOS*	3	0.26	0.03	8.35×10^{-15}	2.01×10^{-10}	Ghrelin opposite strand RNA conserved region|ghrelin opposite strand/antisense RNA|ghrelin/obestatin prepropeptide
cg02913918	*CACNA1E*	1	−0.17	0.02	2.19×10^{-14}	4.96×10^{-10}	Calcium channel, voltage-dependent, R type, alpha 1E subunit
cg07052606	*DCTPP1*	16	0.27	0.04	4.20×10^{-14}	8.98×10^{-10}	dCTP pyrophosphatase 1
cg25660646	*BSG*	19	0.32	0.04	4.85×10^{-14}	9.84×10^{-10}	Basigin (Ok blood group)
cg04007792	*DLGAP2*	8	−0.09	0.01	6.18×10^{-14}	1.19×10^{-9}	Discs, large (*Drosophila*) homolog-associated protein 2

Note: Top 20 CpG sites are based on adjusted p-values, which are listed from smallest p-value to largest p-value. Beta coefficients and p-values were obtained using beta regression (see "Methods" section). Results for all FDR-significant CpGs are provided in Excel Table S1. Table 2 and Excel Table S1 show results for all FDR-significant CpGs. Model-based estimates were derived from beta regression investigating the association between methylation of umbilical cord blood from infants and secondhand smoke exposure from maternal plasma cotinine. These models were adjusted for race/ethnicity, mother's age at delivery, maternal education, and parity, as well as technical covariates (e.g., plate, row, column), and Houseman-estimated cell proportions. The resulting p-values were corrected using Benjamini and Hochberg's FDR method, with CpG sites with FDR <0.05 considered statistically significant. Exposure contrasts for the coefficients indicate change in relation to 1-unit ng/mL increase in cotinine from maternal plasma. "Chr" makes reference to the chromosome number. FDR, false discovery rate; UCSC, University of California, Santa Cruz.

Table 3. Genes with ≥20 significant CpG sites.

Gene name	Chr	# of significant CpG Sites	Function and conditions related to changes in gene
PTPRN2	7	87	Encodes a protein that is an autoantigen in type 1 diabetes (Olsson et al. 2014); regulation of insulin secretion; involvement with insulin-dependent diabetes mellitus as an autoantigen (Li et al. 1997).
MAD1L1	7	46	Component of mitotic spindle-assembly checkpoint that prevents onset of anaphase until all chromosomes are properly aligned at metaphase plate (Ji et al. 2018; Jin et al. 1999; Nakano et al. 2010); this may play role in cell cycle control and tumor suppression (Li et al. 2016).
PRDM16	1	35	Chromosomal aberration involving this gene is found in myelodysplastic syndrome and acute myeloid leukemia (Xinh et al. 2003); functions in the differentiation of brown adipose tissue (Moreno-Navarrete et al. 2018); suppressor of lung adenocarcinoma metastasis (Fei et al. 2019).
ATP11A	13	34	Predictive marker for metachronous metastasis of colorectal cancer (Miyoshi et al. 2010); associated with bronchiectasis (persistent abnormal dilation of the bronchi) and clubbed fingers.
DIP2C	10	33	Loss of DIP2C homolog in rat knock-outs stimulates changes in DNA methylation and epithelial-mesenchymal transition (Larsson et al. 2017); expression in breast cancer (Li et al. 2017).
CAMTA1	1	29	Associated with cerebellar ataxia with mental retardation (neurodevelopmental disorder characterized by mildly delayed psychomotor development, early onset of cerebellar ataxia, and intellectual disability in childhood and adulthood) (Thevenon et al. 2012); immunohistochemical marker for diagnosing epithelioid hemangioendothelioma (Shibuya et al. 2015).
ARHGEF10	8	28	Mutations in this gene are associated with slowed nerve conduction velocity, without any clinical signs of peripheral or central nervous system dysfunction (Verhoeven et al. 2003), and Charcot-Marie-Tooth disease (Boora et al. 2015).
INPP5A	10	28	Mobilizes intracellular calcium and acts as a second messenger mediating cell response to various stimulation; ataxia and cerebellar degeneration in mice (Yang et al. 2015).
TBCD	17	28	Required for correct assembly and maintenance of the mitotic spindle, and proper progression of mitosis; genetic changes can result in early-onset, progressive encephalopathy, and with brain atrophy and thin corpus callosum (an autosomal recessive disease with neurodevelopmental and neurodegenerative features) (Flex et al. 2016).
HDAC4	2	25	Provides instructions for making histone deacetylase 4; involved in the regulation of activity of genes involved in heart and skeletal development and nerve cell survival; mutation in the HDAC4 gene is associated with brachydactyly with no other health problems, intellectual disability, behavioral problems, and skeletal abnormalities (Williams et al. 2010).
RASA3	13	25	Inhibitory regulator of the Ras-cyclic AMP pathway (Molina-Ortiz et al. 2018); functions as a negative regulator of the Ras signaling pathway (Schurmans et al. 2015).
AGAP1	2	24	Functions as a direct regulator of the adaptor-related protein complex 3 on endosomes (Nie et al. 2005); rare variants in this gene have been identified with autism (Pacault et al. 2019).
RPTOR	17	24	Encodes a protein that functions in cell-signaling pathway that responds to nutrient and insulin levels to regulate cell growth (Kim et al. 2002); has a positive role in maintaining cell size and mTOR protein expression, which plays a role in mRNA translation, autophagy, and cell growth; dysregulation of the mTOR pathway has been associated with cancer (Xie and Sun 2019).
SHANK2	11	21	May play a role in the structural and functional organization of the dendritic spine and synaptic function (MacGillavry et al. 2016) and regulate the molecular structure of Shank and the spectrum of Shank-interacting proteins in the postsynaptic densities of the adult and developing brain; mutations in this gene have been associated with autism spectrum disorder (Leblond et al. 2014).
TNXB	6	21	Provides instructions for making tenascin-X, which plays an important role in organizing and maintaining the structure of tissues that support the body's muscles, joints, organs, and skin/connective tissues (Mao et al. 2002); helps to regulate the production and assembly of collagen and elastic fibers in connective tissues (Kolli et al. 2019); mutations in the TNXB gene cause a very small percentage of all cases of a form of Ehlers-Danlos syndrome called the hypermobile type, which is characterized by unusually large range of joint movement (Lao et al. 2020; Micale et al. 2019); a functional variant in the TNXB promoter is associated with the risk of esophageal squamous-cell carcinoma (Yang et al. 2020).
FOXP1	3	20	Has an important role in the regulation of tissue- and cell type-specific gene transcription during both development and adulthood (Liu et al. 2019); may act as a tumor suppressor (Takayama et al. 2014); has been found to promote cancer stem cell-like characteristics in ovarian cancer cells (Choi et al. 2016); mutations resulting in deregulation of its expression plays an important role in the development of lung adenocarcinoma (Sheng et al. 2019) and lymphoma (Goatly et al. 2008); mutations in this gene have resulted in severe intellectual disability syndrome (Meerschaut et al. 2017; Vuillaume et al. 2018) and may play a role in speech and language disorders (Horn et al. 2010; Le Fevre et al. 2013).
SDK1	7	20	Codes for adhesion molecule that promotes lamina-specific synaptic connections in the retina and is expressed in specific subsets of interneurons and retinal ganglion cells (Yamagata et al. 2002); dysregulation of this protein may play an important role in podocyte dysfunction in HIV-associated nephropathy (Kaufman et al. 2004), glomerulosclerosis (Kaufman et al. 2010), and malignant mesothelioma (Cadby et al. 2013).

Note: Chr, chromosome.

When examining significant CpG probes and sorting them based on the number of sites per annotated gene, we found that there were many genes with multiple CpG sites associated with prenatal secondhand smoke exposure (Table 3). We identified 18 genes that had at least 20 statistically significant CpG sites associated with prenatal smoke exposure among nonsmoking mothers. These genes were implicated in insulin regulation (*PTPRN2*, which has the largest number of associated CpG sites at 87); cell signaling and regulation (*MAD1L1*, *INPP5A*, *HDAC4*, *RASA3*, *RPTOR*, *FOXP1*); neuronal processes (*CAMTA1*, *ARHGEF10*, *TBCD*, *SHANK2*); muscle, joint, organ, and/or tissue development (*PRDM16*, *ATP11A*, *DIP2C*, *HDAC4*, *TNXB*, *SDK1*); intellectual disability (*CAMTA1*, *HDAC4*, *FOXP1*), autism (*AGAP1*, *SHANK2*, *FOXP1*); tumor suppression (*MAD1L1*, *FOXP1*); and cancer (*PRDM16*, *RPTOR*, *FOXP1*). Relevant to the potential neurotoxic impact of secondhand smoke is the observation that

the CpG site with the highest beta estimate was cg03343571 of the *RNF39* gene (coefficient $= -1.26$, SE $= 0.19$, adjusted $p = 1.17 \times 10^{-7}$). A total of 19 CpG sites associated with *RNF39* showed significant differential methylation in relation to a 1-unit ng/mL increase in cotinine, with all but three CpG sites (cg26014796, cg22325294, and cg27532187) demonstrating hypomethylation. The *RNF39* gene lies within the major histocompatibility complex class I region of chromosome 6 and has been implicated in multiple sclerosis (Maltby et al. 2017), schizophrenia (Carmel et al. 2020), and allergic rhinitis with and without asthma (Morin et al. 2017).

Functional enrichment analyses revealed several pathways that were enriched in genes associated with prenatal secondhand smoke exposure. Table S1 shows the top 20 pathways identified using the KEGG (Kanehisa and Goto 2000; Kanehisa et al. 2019). These pathways included neuronal signaling (e.g., cholinergic synapse, dopaminergic synapse), metabolism (e.g., gastric acid secretion, insulin secretion, aldosterone synthesis and secretion, thyroid hormone signaling), cell signaling and regulation (e.g., PI3K-Akt signaling, sphingolipid signaling, tight junction, cGMP-PKG signaling, synaptic vesicle cycle, focal adhesion, Wnt signaling), and cancer pathways [e.g., proteoglycans in cancer, pathways in cancer, extracellular matrix (ECM)-receptor interaction]. The findings highlight several pathways relevant to neurodevelopment, metabolic regulation, and cancer that are potentially affected by prenatal secondhand smoke exposure.

Epigenomic enrichment analyses indicated that there was strong enrichment within a specific class of enhancers associated with transcription start sites (TSSs), which affect protein-coding genes (Figure 1A). Notably, no other significant enrichment was observed. The low-level tobacco exposure CpGs were significantly depleted in permissive, robust, and cell type-specific enhancers (Excel Table S2 shows FANTOM Annotations from GenomeRunner) and in long noncoding RNAs and pseudogenes but were enriched in protein-coding genes (Figure 1B). Furthermore, CpGs were enriched in RNA Polymerase II binding sites (transcribing protein-coding genes) but were depleted in RNA Polymerase III (transcribing noncoding genes) (Table S2 shows Ensembl Gene Classes from GenomeRunner, and Excel Table S3 shows Transcription Factor Binding Sites from GenomeRunner). Similar to the functional enrichment pathway analysis, this analysis identified metabolic pathways, immune-related signaling, cancer pathways, and neuronal pathways among the most significant (Excel Table S4 shows KEGG Enriched Pathways). These results suggest that low-level tobacco smoke exposure may affect CpGs located near protein-coding genes and that such exposure may have a direct effect on the proteome in such a way as to influence transcription and translation of proteins.

Independent Validation of Associations between Cotinine Levels and DNA Methylation

As shown in Table 4, independent analysis of *AGER* in these participants failed to replicate the findings from the 450K BeadChip analyses ($n = 101$; cg09199225; estimate $= -0.16$, SE $= 0.21$, $p = 0.439$). However, the results for *PRKG1* from the 450K BeadChip analyses were replicated for cg17079497 in the independent group of participants, with 1-unit ng/mL increase in cotinine significantly inversely correlated with the level of methylation at this CpG site ($n = 115$, estimate $= -1.09$, SE $= 0.45$, $p = 0.018$). As shown in Table S3, we found cg17079497 associated with *PRKG1* to be significantly correlated with maternal cotinine in plasma values (correlation $= -0.20$, $p = 0.03$). Quantile plots, showing associations between pyrosequencing markers (e.g., cg20720326 and cg09199225 associated with *AGER* and cg17079497 and cg22899538 associated with *PRKG1*) and maternal cotinine in plasma are shown in Figure S2.

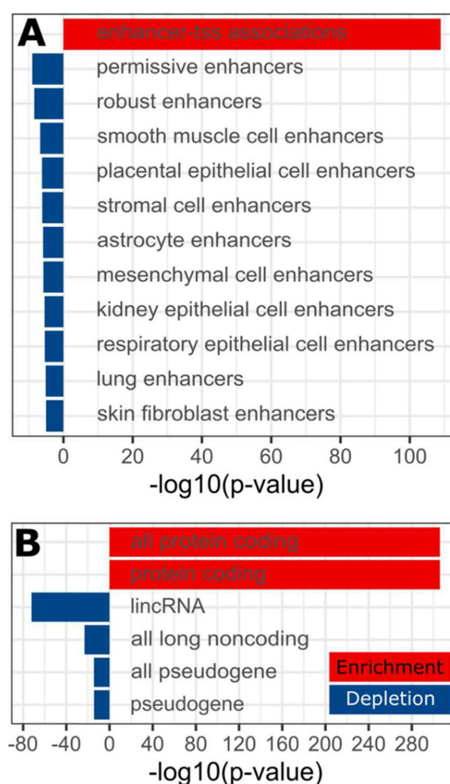

Figure 1. Epigenomic analysis of cotinine associated CpG sites.

Discussion

Similar to studies assessing active smoking during pregnancy, our results suggest that secondhand smoke exposure, measured via cotinine in maternal plasma, among a sample of nonsmoking pregnant women was significantly associated with alterations in DNA methylation of infant cord blood. In this EWAS study, we found 29,049 CpG sites to be differentially methylated in relation to a 1-unit ng/mL increase in cotinine among self-reported nonsmoking mothers. A number of these CpG sites were found within genes implicated in neuronal signaling, cell signaling and regulation, metabolic pathways, and cancer pathways. Further, epigenomic enrichment analyses showed a potential vulnerability to disruption in metabolic and immune-related development in response to prenatal secondhand smoke exposure. We also were able to replicate significant results from the 450K BeadChip using another independent subsample of the NEST cohort for the *PRKG1* gene. Overall, results suggest that even low levels of secondhand smoke exposure during pregnancy, which likely represents exposure to other family and/or community members who smoke in the women's environments, is related to alterations in DNA methylation of umbilical cord blood.

Methylation of CpG Sites in Relation to Low-Level Tobacco Smoke Exposure

Methylation of CpG sites associated with *AHRR* have been used to predict smoking status with high accuracy (Reese et al. 2017; Richmond et al. 2018; Tehranifar et al. 2018). Blood *AHRR* methylation at cg0557592 is inversely associated with smoking (Bojesen et al. 2017; Reynolds et al. 2015) and lung cancer (Bojesen et al. 2017), suggesting that *AHRR* may mediate the metabolism of xenobiotic particles like cigarette smoke. However,

Table 4. Comparison of 450K and pyrosequencing analyses.

Gene	CpG Site	Chr	Position	450K Beadchip analyses Beta	SE	FDR-corrected p-value	Methylation marker	Mean proportion of methylation	Pyrosequencing analyses Beta	SE	Regression p-value
AGER	cg09199225	6	32149260	1.38	0.16	1.19×10^{-13}	cg09199225	92.9	−0.16	0.21	0.439
	cg20720326	6	32152335	0.57	0.09	3.65×10^{-07}	cg20720326	87.8	0.14	0.21	0.490
	cg26492916	6	32149401	0.12	0.03	3.26×10^{-03}					
	cg17874413	6	32152269	0.08	0.02	1.73×10^{-02}					
	cg27580693	6	32150024	0.08	0.03	4.27×10^{-02}					
PRKG1	cg05396900	10	52750736	0.44	0.05	6.42×10^{-13}					
	cg27380599	10	52834067	0.14	0.03	1.26×10^{-04}					
	cg05402976	10	52993591	−0.08	0.02	2.26×10^{-02}					
	cg24609819	10	52840377	−0.18	0.06	2.47×10^{-02}					
	cg22899538	10	52754927	−0.15	0.05	4.44×10^{-02}	cg22899538	90.3	−0.49	0.47	0.297
	cg17079497	10	53655621	−0.14	0.05	4.61×10^{-02}	cg17079497	84.5	−1.09	0.45	0.018

Note: Beta coefficients and p-values were obtained using beta regression for 450K Beadchip analyses (see "Methods" section). Only CpG sites that were significant for 450K Beadchip analyses at FDR $p<0.05$ are listed for *AGER* and *PRKG1*. Linear regression p-values for pyrosequencing analyses are not FDR-corrected. Cotinine values under the LOD were recoded to 0 ng/mL for both 450K Beadchip analyses ($n=0$) and pyrosequencing analyses ($n=3$). Chr, chromosome; FDR, false discovery rate; LOD, level of detection; SE, standard error.

these studies did not examine associations in nonsmokers. In our study, which focuses on nonsmokers, we found altered methylation at 10 CpG sites associated with the *AHRR* gene in relation to low levels of secondhand smoke exposure. This finding suggests that DNA methylation of *AHRR* in infant cord blood (or from young children) may also be a useful surrogate marker of secondhand smoke exposure when direct exposure cannot be established. Of note, however, was the lack of significance for the CpG probe most often associated with smoking status, that of cg05575921 associated with *AHRR* (Bojesen et al. 2017; Joubert et al. 2012; Philibert et al. 2020, 2012). We did identify other CpG sites within AHRR showing significant differences in methylation in infant cord blood associated with secondhand smoke exposure during pregnancy, including one in 509 base pairs of cg05575921 (e.g., cg22103736, beta = 0.15, SE = 0.03, adjusted $p=8.6\times10^{-4}$). All of the significant probes across this region showed hypermethylation with smoke exposure (see Excel Table S1), which contrasts with the inverse correlation between methylation and smoke exposure previously reported. It is not clear why there is a directional difference in effect, but results may indicate that low-level indirect exposure elicits different effects on DNA methylation than higher-level exposure, as is the case for babies born to mothers who smoked during pregnancy. Further, the vulnerability of this region of the genome at *AHRR* to tobacco smoke [and to cannabis smoke exposure, see (Murphy et al. 2018)] suggests that there is inherent plasticity of the *AHRR* epigenome at multiple regions that may be useful for monitoring history of different levels of tobacco smoke exposure.

Six CpG sites associated with the gene *PRKG1* were methylated in umbilical cord blood in relation to elevated cotinine levels in maternal plasma (Table 4). Of particular interest was cg17079497, which demonstrated statistically significant hypomethylation with both the 450K BeadChip analysis and in an independent subset of the NEST cohort by pyrosequencing. *PRKG1* isoforms act as key mediators of the nitric oxide/cGMP signaling pathway and are important components of many signal transduction processes in diverse cell types. The well-studied homolog of *PRKG1*, foraging (*for*) gene in *Drosophila melanogaster* encodes a cGMP-dependent protein kinase (PKG). PKG activity has been found to interact with early-life stress in determining adult exploratory (i.e., the tendency to investigate a novel area) and fitness traits (i.e., fecundity) (Burns et al. 2012). Recent studies have also demonstrated genotypic variation in *PRKG1* is associated with unique patterns of self-regulation in humans, such as cautious exploration of novel environments (Struk et al. 2019). Further, significant interactions between the *PRKG1* C allele variant of rs1729578 and trauma exposure in relation to alcohol misuse symptoms in humans (Hawn et al. 2018; Polimanti et al. 2018), provides support for the potential role of *PRKG1* in stress response-related traits in humans.

Many of the other CpGs are consistent with what has been reported in other studies examining differential methylation in relation to maternal self-reported smoking during pregnancy. Of note is one CpG site that overlapped between our study and that conducted by Joubert et al. (2016) (e.g., cg18316974 associated with *GFI1*). There were six FDR-significant CpGs in *GFI1* associated with smoke exposure in our population. Of these CpGs, four were hypermethylated. Thus, secondhand smoke exposure was not found to be generally associated with hypermethylation in *GFI1*, in contrast with previous findings for sustained maternal smoking during pregnancy (de Vocht et al. 2015; Küpers et al. 2015). Results are consistent with prior studies indicating differential methylation of CpG sites associated with *GFI1* between smokers and nonsmokers (Parmar et al. 2018; Philibert et al. 2013; Wan et al. 2012; Zeilinger et al. 2013). *GFI1* has been found to play a role in developmental disorders; it is associated with birth weight (Küpers et al. 2015), hematopoiesis, and decreased body mass index and waist circumference (Parmar et al. 2018); and it is involved in oncogenesis (Küpers et al. 2015).

As with other environmental epigenetic studies (Reynolds et al. 2017), the effect sizes that we find in our study are small (see Figure S2). As such, the ability to detect differences in the validation cohort is limited, particularly if there was more variability in the validation cohort in the methylation levels measured across these specific CpGs. Nevertheless, small effect sizes associated with exposure are common among environmental epigenetic studies. Breton et al. (2017) posit that larger effect sizes, such as that observed in cancer, are less common because large shifts may be incompatible with continued development. The dynamic nature of the epigenome emphasizes the importance of longitudinal studies, which allow for profiling of the epigenome over both time and changing environmental exposures. Longitudinal studies will also help to improve our ability to identify small changes and determine the effect of consistent changes across time (Breton et al. 2017).

Functional Interpretation of Differentially Methylated Genes

We performed enrichment analysis to facilitate the functional interpretation of our differentially methylated genes. Pathway analysis indicated enrichment of CpG sites corresponding to genes involved in biological processes related to metabolic regulation, neuronal signaling, cell signaling and regulation, and

cancer pathways. Common across these pathways is the mitogen-activated protein kinase (MAPK) signaling pathway, which plays an important role in cerebrovascular receptor plasticity (Cseh et al. 2014; Rauen 2013), as well as the regulation of gene expression, cellular growth, and survival (Knight and Irving 2014). Exposure to cigarette smoke has been shown to activate signaling pathways in airway epithelial cells, including the MAPK signaling pathway (Xu et al. 2015). Abnormal MAPK signaling may lead to increased or uncontrolled cell proliferation, resistance to apoptosis, and resistance to chemotherapy, radiotherapy, and targeted therapies through abnormal expression of pathway receptors and/or genetic mutations—all of which play an important role in the development of some cancers (Santarpia et al. 2012). This finding suggests that the MAPK pathway may be one of the mechanism by which exposure to secondhand smoke during pregnancy increases risk for cancer among offspring (Metayer et al. 2013; White et al. 2017).

Interpretation of Epigenomic Enrichment within Protein-Coding Genes Associated with Obesity-Related Traits and Obesity and Immune-Related Pathways

Similar to another study investigating enrichment analysis for the genomic features of cigarette smoking (Joehanes et al. 2016), our study demonstrated strong enrichment of CpG sites within enhancers among infants exposed to secondhand tobacco smoke *in utero*. Specifically, enhancers associated with transcription start sites (TSS) were affected, which supports the potential direct impact of secondhand smoke exposure on gene expression. Prior research has demonstrated that enhancers are more susceptible to methylation changes (Ziller et al. 2013) relative to other genomic regions, such as CpG islands, that are more resistant to abrupt changes in DNA methylation in response to environmental exposures (Ivanova et al. 2012). Thus, in combination with pathway analysis, our results suggest that the secondhand smoke exposure-associated CpG sites may have regulatory effects on several disease processes affecting metabolism, immunity, and cancer-related pathways.

Limitations

This prospective study should be considered in light of certain strengths and limitations. Strengths include use of a sample that includes both Black and non-Hispanic White women and their children; the use of an established, highly reproducible methylation array; and cotinine measures from maternal blood samples drawn during pregnancy to assess secondhand smoke exposure. One potential limitation of this study is related to exposure assessment. Plasma cotinine reflects a snapshot of exposure during pregnancy and may not accurately characterize exposure over the course of pregnancy or at different periods during pregnancy. Additionally, future larger studies may want to quantify varying cut points of quantitative smoke exposure to determine threshold effects on the methylation of the epigenome. Another potential limitation involves variability in DNA methylation profiles between human tissue/cell types. For this study, we used genomic DNA isolated from umbilical cord blood leukocytes. Blood samples contain interindividual variability in the number of different kinds of DNA-containing cells. Prior studies have demonstrated that disparities in methylation can arise due to the variability in cell composition when blood is used in DNA methylation analysis studies (Joubert et al. 2012; Monick et al. 2012). However, our study results show minor difference in results when adjusting for Houseman cell compositions suggesting that variability in DNA methylation due to cell composition is probably small. Another potential concern is the presence of confounding by genetic variability, which can be exacerbated in studies with small sample sizes. In addition, as is the case with many EWAS studies, the 450K BeadChip array provides measurements for >480,000 CpG sites across the genome representing 99% of the RefSeq genes and 96% of CpG islands; density of CpG site coverage is still limited to the CpG sites included in the array and does not provide information on neighboring sites. Also, it is possible that the results of 450K BeadChip analyses vary between different arrays (e.g., batch effects, which were adjusted for in these analyses) or may not reflect true DNA methylation due to problematic probes and intricacies in data normalization and/or data analyses. Further, the Illumina 450K BeadChip platform may have probes that do not perform well and thus have the potential to produce erroneous results (Sugden et al. 2020). To overcome these potential limitations, next-generation sequencing can be used on CpG sites detected by the 450K BeadChip to verify changes in DNA methylation status and assess neighboring CpG sites contained within a range of specified parameters (Miyake et al. 2018). Additionally, it is possible that copy number variants (CNVs) or single nucleotide polymorphisms (SNPs) could be contributing to results. It is unlikely that SNPs are driving results, however, because CpG sites known to harbor common SNPs were excluded in analyses. It is more difficult to determine the effect of CNVs without doing a full genome assessment for copy number changes in the specimens tested, and it is unclear how common CNVs are in umbilical cord blood—suggesting that further research is needed. Finally, although our statistical models did control for race/ethnicity, age, parity, and maternal education, there is the potential for confounding by other factors (e.g., socioeconomic status, stress, diet, and alcohol consumption) that are warranted for inclusion in future follow-on studies.

Conclusion

In summary, we observed evidence of significant differences in the DNA methylation of umbilical cord blood across individual CpGs, as a function of secondhand tobacco smoke exposure measured by cotinine concentration among nonsmoking mothers during pregnancy. Our study provides preliminary evidence of effects of secondhand smoke exposure on DNA methylation in umbilical cord blood and suggests underlying molecular mechanisms that might be affected. These findings further highlight potentially harmful effects on the epigenome of babies born to nonsmoking pregnant women who are exposed to tobacco smoke from family members or in the community. Future studies are needed to replicate these findings in other study populations. Further replication would support the importance of eliminating secondhand smoke exposure during pregnancy through home and community smoke-free policies.

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B.F.F. was PI for project 1 of the NICHES study and involved in the data acquisition, the study's focus on secondhand smoke

exposure and DNA methylation, design of the study, interpretation of results, and contributed to drafting and revising the manuscript. M.G.D. performed bioinformatics/statistical analyses of methylation data, and generated results tables and figures. E.K.D. contributed to the statistical analysis of pyrosequencing data, as well as conducting background literature for the introduction and the development of the results, methods, and discussion. C.G. and Z.H. facilitated sample preparation for 450K BeadChip data generation and generated the pyrosequencing data. S.K.M. was involved in study conception and design, data interpretation, and contributed to drafting and revising the manuscript. J.Z. supervised cotinine assays and reviewed the manuscript. C.H. was the PI and was involved with the design of the NEST cohort. S.H.K. was involved in the data acquisition of the NICHES study. All of the authors discussed the results, contributed to the discussion, and helped in the preparing and editing of this manuscript.

Study data is available from the corresponding author (B.F.F.) upon reasonable request.

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