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Article

Prenatal Smoking Exposures and Epigenome-Wide Methylation in Newborn Blood

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ABSTRACT: **BACKGROUND**: Maternal sustained smoking during pregnancy is associated with thousands of differentially methylated CpGs in newborns, but impacts of other prenatal tobacco smoking exposures remain unclear. **OBJECTIVE**: To identify differential DNA methylation in newborns from maternal sustained smoking and less studied prenatal smoking exposures (i.e., maternal exposure to secondhand smoke [SHS] exposure during pregnancy, maternal quitting before pregnancy, paternal smoking around conception, and paternal quitting before pregnancy). **METHODS**: We conducted a large meta-analysis of prenatal tobacco smoking exposures and epigenome-wide newborn blood DNA methylation through the Pregnancy And Childhood Epigenetics Consortium (PACE). Across 19 cohorts, 11,175 parent-newborn pairs contributed information on at least one prenatal smoking exposure, mostly from questionnaires. Maternal blood or urine cotinine measurements, available in a few studies, provided objective data for maternal SHS and smoking during pregnancy. Primary analyses used Illumina450 K methylation data; secondary analyses in 5 cohorts examined CpGs unique to the EPIC array. **RESULTS**: Maternal sustained smoking associated with differential DNA methylation (false discovery rate [FDR] < 0.05) at 8,862 CpGs on the 450 K (n = 8,148) and did not differ by infant sex. We identified over 300 novel genes not previously identified in EWAS of smoking. No differential methylation was associated with maternal SHS, maternal former smoking, or paternal smoking around conception. However, cg24805739 (*MED13L*) was associated with former paternal former smoking. Forty-one novel genes were identified using maternal cotinine measurements compared to questionnaire. In EPIC unique analyses (n = 3,415), differential methylation was observed with maternal sustained smoking (211 CpGs), maternal SHS (5 CpGs), and paternal former smoking (4 CpGs). Smoking-associated CpGs in blood were strongly enriched for functional elements across multiple tissues. **CONCLUSIONS**: Maternal sustained smoking has the largest impact on newborn DNA methylation, suggesting a strong influence of the intrauterine environment. We observed minimal impacts for less studied exposures including SHS, maternal former smoking, and paternal smoking.

BACKGROUND

Parental tobacco smoking exposure has numerous adverse effects on offspring health. For example, maternal smoking during pregnancy has been causally linked to lower infant birth weight and increased early respiratory illness and is associated with many other adverse health outcomes in children.[1] Maternal exposure to secondhand smoke (SHS) during pregnancy has been reported to increase risk of stillbirths and congenital malformations.[2] There is also emerging evidence that paternal smoking can independently influence outcomes in the offspring, including congenital malformations and childhood leukemia.[3] Alterations in newborn DNA methylation may contribute to those adverse health outcomes.

The Pregnancy And Childhood Epigenetics (PACE) consortium identified extensive differential DNA methylation

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Table 1. Characteristics of Participating Studies

Study	Methylation Array	Years of Birth	No. of Newborns by Ancestry or Race/Ethnicity				No. Exposed by Smoking Exposure[a]				
			European	African	Hispanic/Latino	Maternal Sustained Smoking	Maternal SHS[b]	Maternal Former Smoker[b]	Paternal Smoking Around Conception[b]	Paternal Former Smoker[b]	Maternal Cotinine
ALSPAC	450 K	1991–1992	820			66	160	47	108	105	156
CCLS1	450 K	1982–2008	146		116		52	52		54	
CCLS2	450 K	1982–2008	88		149		56	35	27	19	
DCHS	450 K	2012–2015		119[c]		41					97
EDEN	450 K	2003	161			21	15		31	48	
ENVIRONAGE	450 K	2014–2015	175			22		30			
Gen R	450 K	2002–2006	1,124			146	315	214	276		
GECKO	450 K	2006–2007	255			127		34		32	
Healthy START	450 K	2009–2014	314	88	146	23	58	69			
INMA	450 K	2003–2008	377			56	138	86	69		169
LiNA	450 K	2006–2008	472				103		30	19	
MoBa1	450 K	2002–2004	1051			148	125	214	163	100	852
MoBa2	450 K	2000–2005	681			76	92	123	110	63	183
NCL	450 K	1996–2001	890			233	322		103	133	
NEST	450 K	2005–2009	155	164		35	21				30
POSEIDON	450 K	2010–2017	269			30	15	86	42	44	
Total 450 K			6978	371	411						1487
CCLS4	EPIC	1982–2014	104		181		51	41	24	51	
DCHS	EPIC	2012–2014		148[3]		38					134
ENVIRONAGE	EPIC	2010–2015	325			34		61			
MoBa-START	EPIC	2000–2008	2,000			106	184	446	256	349	
Upstate KIDS	EPIC	2008–2010	657			28	60	155			
Total EPIC			3086	148	181						134
Total 450 K + EPIC			10064	519	592						

[a]Numbers provided for studies that were included in the meta-analysis. Additional details available in Table S2. [b]Among pregnant participants who did not smoke during pregnancy. [c]~50% admixed.

in newborn blood related to maternal smoking during pregnancy and observed that signals were stronger when mothers continued smoking throughout pregnancy.[4] It remains unclear whether other prenatal tobacco smoking exposures (e.g., maternal SHS exposure, mothers who quit smoking before pregnancy [i.e., maternal former smokers], and paternal smoking exposures [i.e., paternal smoking around conception, paternal former smoking]) are associated with newborn DNA methylation.[5] Additionally, the majority of studies that have identified differential DNA methylation in relation to maternal smoking during pregnancy classified smoking status based on questionnaire data and the known underreporting of smoking is more pronounced in pregnant women compared to nonpregnant women.[6,7] Cotinine measured during pregnancy is an objective biomarker of smoking status that identifies false reporting of nonsmoking and, when high sensitivity assays are used, can detect SHS exposure.

In this large multicohort study, we evaluated a comprehensive range of prenatal tobacco smoking exposures based on questionnaire and maternal cotinine measurements in relation to epigenome-wide newborn blood DNA methylation.

METHODS

Study Populations

A total of 11,175 parent-newborn pairs across 19 cohorts had information on at least one prenatal tobacco smoking exposure (Table 1). Participating cohorts included Avon Longitudinal Study of Parents and Children (ALSPAC),[8,9] California Childhood Leukemia Study (CCLS1, CCLS2, CCLS4),[10] Drakenstein Child Health Study (DCHS),[11] Étude des Déterminants pré et post natals de développement et de la santé de l'ENfant (EDEN),[12] The ENVIRonmental influence ON early AGEing (ENVIRONAGE),[13] Generation R Study,[14] Groningen Expert Center for Kids with Obesity (GECKO),[15] Healthy Start Study,[16] INfancia y Medio Ambiente (INMA),[17] Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk (LINA),[18] Norwegian Mother, Father and Child Cohort Study (MoBa1, MoBa2, MoBa-Study of Assisted Reproductive Technology [MoBa-START]),[19,20] Norway Facial Clefts Study (NCL),[21] Newborn Epigenetics Study Cohort (NEST),[22] Pre-, peri- and pOstnatal Stress in human and nonhuman offspring: A translational approach to study the Epigenetic Impact on DepressiON (POSEIDON),[23,24] and Upstate KIDS Study.[25] Thirteen cohorts enrolled women during pregnancy (ALSPAC, DCHS, EDEN, GECKO, Generation R, Healthy Start, INMA, LiNA, MoBa1, MoBa2, MoBa-START, NEST, POSEIDON) and six enrolled at or after birth (CCLS1, CCLS2, CCLS4, ENVIRONAGE, NCL, Upstate KIDS). Each cohort obtained approval to conduct human subjects' research by their appropriate review board(s), and written consent was provided by the enrolled adult.

Twelve of these 19 cohorts (n = 6,035) did not participate in the previous PACE smoking project:[4] CCLS1, CCLS2, CCLS4, DCHS, EDEN, ENVIRONAGE, Healthy Start Study, INMA, LINA, MoBa-START, POSEIDON, Upstate KIDS. Women in DCHS had African ancestry or were admixed. In the CCLS cohorts and Healthy Start, self-reported racial/ethnic group (non-Hispanic white, Hispanic, and African American) was used as a proxy for genetic ancestry. Women in all other cohorts were of self-reported non-Hispanic white or European ancestry. Additional details of each study can be found in the Supplemental Methods. Cotinine during pregnancy was measured in 1,843 women across in seven studies: three in plasma (MoBa1, MoBa2, NEST) and three in urine (ALSPAC, DCHS, Healthy Start, INMA). However, due to a limited number of smokers, Healthy Start was not included in the cotinine analyses. One cohort (ALSPAC)

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measured cotinine during the first trimester, and the other five measured cotinine in the second or third trimester (Table S1). Each cohort used a different assay to measure cotinine (Table S1).

Prenatal Tobacco Smoking Exposures

Pregnant participants self-reported their smoking exposures in cohort-specific questionnaires. Based on the findings from the previous PACE smoking paper,[4] women were categorized as never smoked during pregnancy, quit smoking in early pregnancy, or continued smoking throughout pregnancy (e.g., sustained smoking). To evaluate a potential dose response relationship, we created an ordinal variable coding nonsmokers as 0, sustained smokers reporting 1−4 cigarettes/day as 1 and 5+ cigarettes/day as 2. Women who reported being exposed ≥1 h/day to SHS at home or work or if the father smoked and lived with the mother were classified as exposed to SHS and unexposed otherwise. In 16 cohorts (n = 9,538), we were able to further disaggregate women who never smoked during pregnancy into those who never smoked in their lifetime versus former smokers who quit before conception. Six cohorts had data on cotinine levels collected using different analytic platforms and biological matrices and thus had different cutoffs indicative of active smoking (Table S1). Women were categorized as sustained smokers regardless of questionnaire responses if their cotinine level was indicative of active smoking at the time of the measurement.

Paternal smoking was self-reported by either the pregnant participant or the father. Fathers who quit smoking during pregnancy or still smoked during pregnancy were considered to have smoked around conception, and those who did not report smoking during this pregnancy were considered to have not smoked around conception. Data in 12 cohorts (n = 7,383) allowed us to further disaggregate fathers who never smoked in their lifetime and those who quit before conception (i.e., former smokers).

DNA Methylation Measurement and Quality Control

Using each cohort's preferred laboratory, DNA was isolated from newborn blood (either cord blood or heel stick), and DNA methylation was measured in newborns using Illumina's 450 K array or EPIC array. Each cohort applied quality control and normalization procedures as described in the supplemental methods. Studies corrected for batch effects by either using ComBat[26] or including surrogate variables, principal components, or plate as a covariate. Methylation data were analyzed by using β-values. To reduce the impact of extreme outliers in the methylation data for each CpG, β-values that were outside three times the interquartile range below the 25th percentile or above the 75th percentile were removed by individual cohorts or a 99% winsorization was performed.

Cohort Specific Epigenome-Wide Analyses

Cohorts ran epigenome-wide analyses using robust linear regression in R, where smoking exposure was the independent variable and DNA methylation was the outcome. We conducted the following epigenome-wide analyses restricted to newborns with mothers who did not smoke during pregnancy: maternal exposure to SHS during pregnancy (yes/no), maternal former smoker versus never smoker, paternal smoking around conception (yes/no), and paternal former smoker versus never smoker. The cohorts conducted epigenome-wide analyses for each prenatal smoking exposure where there were at least 15 exposed women. Two cohorts (DCHS, ENVIRONAGE) measured DNA methylation in subsets of their study population using different methylation arrays, so analyses were conducted separately by array (450 K or EPIC). Cohorts that measured DNA methylation in diverse populations performed separate analyses for each ancestry or race/ethnicity group. Analyses were not conducted for specific ancestry or race/ethnicity groups when there were less than 15 exposed.

To objectively capture the amount of maternal smoking exposure and to identify nonsmokers more accurately during pregnancy, studies with maternal cotinine data ran epigenome-wide analyses of cotinine (modeled continuously) and newborn DNA methylation. These cohorts also repeated the analyses restricted to women who did not smoke during pregnancy to evaluate whether exposure in the SHS range is associated with DNA methylation.

Covariates were either self-reported from the enrolled person, abstracted from clinical or birth certificate records, or calculated from methylation data. All studies adjusted for maternal age at delivery, maternal education (2−4 categories based on the availability information and distribution in the cohort, as a proxy for socioeconomic status), parity (nulliparous vs parous), gestational age at birth, maternal prepregnancy body mass index, infant sex, and 7 cell type proportions (nRBC, CD8T, CD4T, NK, B cell, monocytes, granulocytes). Cell type proportions were estimated using the Houseman method with the combined reference panel for cord blood and the IDOL optimization.[27,28] Cohort-specific covariates included technical variables (e.g., batch) and selection factors (i.e., characteristics for which participants were selected (e.g., childhood asthma status)), where appropriate. See the Supporting Information and Table S2 for cohort specific details.

Quality Control of Individual Cohorts

Lambdas were calculated for each model. For each exposure, the median standard error and square root of the number exposed from each cohort was plotted to check that studies with larger sample size had smaller standard errors. We also visualized the distribution of beta coefficients and standard errors across studies by generating boxplots for each model. Because the median cohort-specific standard error ranged from 0 to 0.006, CpGs with standard errors greater than 1 were excluded from meta-analyses. cg05575921 (*AHRR*) is a well-established CpG related to maternal sustained smoking;[4] thus, we checked that this CpG was at least nominally significant in the cohort-specific results for maternal sustained smoking. In the NEST results for European ancestry, none of the top 10 CpGs had been associated with smoking in the EWAS Catalog, a database which contains results from large smoking meta-analyses.[4,29] Concerned that these results might be outliers, we excluded this subpopulation (n = 155) from all meta-analyses.

Meta-Analysis and Probe Filtering

For dichotomous exposure variables, fixed effects meta-analysis of study-specific results was performed using the tool, METAL, with inverse-variance weighting.[30] Because cotinine was measured in plasma and urine, separate meta-analyses were conducted by a biological matrix, weighting the effects by sample size. There were 16 data sets (n = 7,760) that measured DNA methylation using the 450 K array and five data sets that used the EPIC array (n = 3,415) (Table 1); thus, the primary meta-analysis and downstream analyses focused on results for CpGs on the 450 K array, including studies that measured methylation on the EPIC array. Secondary analyses were conducted for CpGs only on the EPIC array. Up to 431,853 CpGs on the 450 K array and 376,377 CpGs unique to the EPIC array on the autosomal chromosomes were meta-analyzed. The cohort-specific results were meta-analyzed at the National Institute of Environmental Health Sciences, and a shadow meta-analysis was conducted independently at ISGlobal, Barcelona. After meta-analysis, CpGs previously reported to be potentially problematic (i.e., "ch" probes, probes with a SNP in the extension base that can cause color channel switch, probes with extension base inconsistencies, and cross-reactive probes)[31,32] were removed. CpGs were considered differentially methylated using false discovery rate (FDR < 0.05).[33] For FDR significant CpGs, leave-one-out analyses were conducted for meta-analyses with at least 5 cohorts. CpGs that were not nominally significant after leave-one-out analyses were excluded. Where there were 5 cohorts or fewer with data for FDR significant CpGs, forest plots were generated.

Enrichment of Functional Genomic Features

We used eFORGE v2.0 to identify tissue- and cell type-specific enrichment for DNase I hotspots, 15 chromatin states, and five histone marks.[34−36] The top 1000 CpGs were compared to background CpGs from the 450 K array, selecting background bins and adjusting for gene relationship and CpG island relationship.

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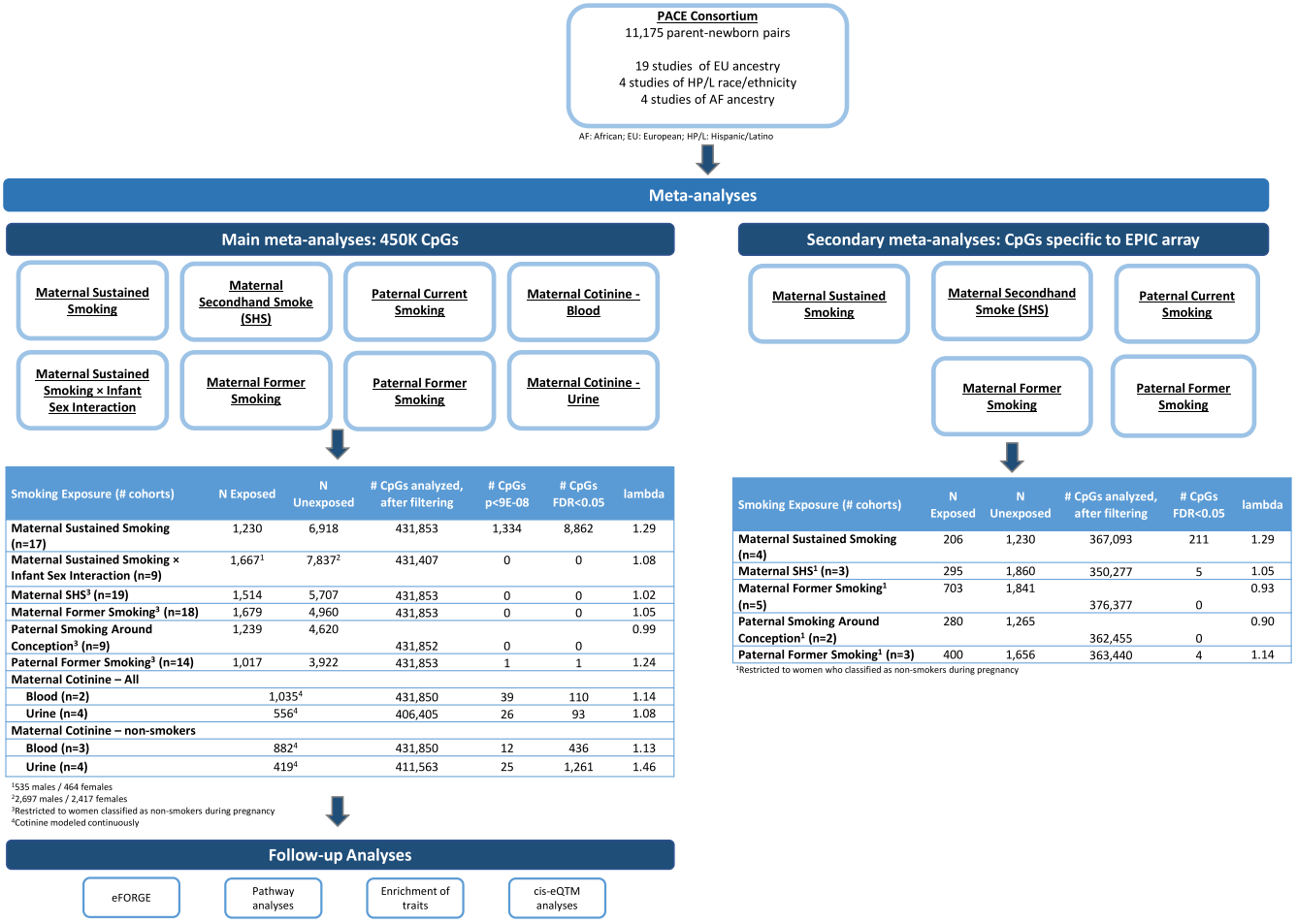

Smoking Exposure (# cohorts)	N Exposed	N Unexposed	# CpGs analyzed, after filtering	# CpGs p<9E-08	# CpGs FDR<0.05	lambda
Maternal Sustained Smoking (n=17)	1,230	6,918	431,853	1,334	8,862	1.29
Maternal Sustained Smoking × Infant Sex Interaction (n=9)	1,667[1]	7,837[2]	431,407	0	0	1.08
Maternal SHS[3] (n=19)	1,514	5,707	431,853	0	0	1.02
Maternal Former Smoking[3] (n=18)	1,679	4,960	431,853	0	0	1.05
Paternal Smoking Around Conception[3] (n=9)	1,239	4,620	431,852	0	0	0.99
Paternal Former Smoking[3] (n=14)	1,017	3,922	431,853	1	1	1.24
Maternal Cotinine – All						
Blood (n=2)	1,035[4]		431,850	39	110	1.14
Urine (n=4)	556[4]		406,405	26	93	1.08
Maternal Cotinine – non-smokers						
Blood (n=3)	882[4]		431,850	12	436	1.13
Urine (n=4)	419[4]		411,563	25	1,261	1.46

[1]535 males / 464 females
[2]2,697 males / 2,417 females
[3]Restricted to women classified as non-smokers during pregnancy
[4]Cotinine modeled continuously

Smoking Exposure (# cohorts)	N Exposed	N Unexposed	# CpGs analyzed, after filtering	# CpGs FDR<0.05	lambda
Maternal Sustained Smoking (n=4)	206	1,230	367,093	211	1.29
Maternal SHS[1] (n=3)	295	1,860	350,277	5	1.05
Maternal Former Smoking[1] (n=5)	703	1,841	376,377	0	0.93
Paternal Smoking Around Conception[1] (n=2)	280	1,265	362,455	0	0.90
Paternal Former Smoking[1] (n=3)	400	1,656	363,440	4	1.14

[1]Restricted to women who classified as non-smokers during pregnancy

Figure 1. Diagram summarizing analyses and meta-analyses results.

Pathway Analyses

CpGs were annotated to genes using Illumina's manifest (hg19). The full epigenome-wide results after excluding problematic probes were used in pathway analyses using the "methylGSA" package in R. Robust rank aggregation[37] accounted for the probe number bias (i.e., differing number of CpGs per gene).[38] We applied the Gene Set Enrichment Analysis (GSEA)[39] with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[40,41] and considered pathways with gene sets between 50 (to include the small cell lung cancer pathway) and 500 genes (the default).

Enrichment of Traits in EWAS Atlas

EWAS Toolkit conducts enrichment analyses of inputted CpGs against the data in EWAS Atlas, a database of results from epigenome-wide association studies.[42] We input the top 1000 CpGs from the four exposures to identify traits enriched with our findings.

Associations with Gene Expression

The FDR significant CpGs from the primary meta-analyses (450 K), were looked up in a published study that reported the association between blood DNA methylation and *cis* gene expression in 832 children from the Human Early Life Exposome (HELIX) project,[43] as well as in meta-analysis of 3,075 adults in the Biobank-based integrative omics study (BIOS) consortium from the following four cohorts: Leiden Longevity Study, LifeLines Study, Rotterdam Study, and Netherlands Twin Register.[61] The genes from RNaseq data were annotated based on GRCh 37 using R package biomaRt.[44] Raw count data were transformed to log2CPM using the voom function in the limma package.[62] DNA methylation data were annotated using the Illumina hg19 annotation file for 450 K array. cis-eQTMs for significant CpG sites were conducted for gene expression transcripts within ±250 kb of each CpG site by linear regression model using R package limma.[62] The final model was: gene expression (log2CPM) ~ DNA methylation (M values) + age + sex + lymphocyte proportion + monocyte proportion + granulocyte proportion + RNA flow cell number. The model inflation was corrected using the "bacon" method.[45] cis-eQTM analyses were performed for each cohort, and the results were meta-analyzed using an inverse variance-weighted fixed-effects model in METAL.[30] Correction for multiple testing based on number of CpG sites was performed using Benjamini-Hochberg.[33]

RESULTS

Exposures analyzed and a summary of their observed results are displayed in Figure 1. For each of the 7 estimated cell types, mean cell proportions, weighted by cohort sample size, were nearly identical between newborns whose mothers did not smoke during pregnancy and those whose mothers were sustained smokers. The weighted mean cell type proportions were the same for nRBC (0.13), NK (0.05), B-cell (0.05), and monocytes (0.07) and very slightly differed for CD8T (nonsmoker: 0.06; sustained smoker: 0.05), CD4T (nonsmoker: 0.13; sustained smoker: 0.12), and granulocytes (nonsmoker: 0.52; sustained smoker: 0.54). Details of the study characteristics can be found in Tables S1−S3.

Maternal Sustained Smoking

In a meta-analysis of 17 cohorts, comparing 1,230 newborns whose mothers were sustained smokers during pregnancy to 6,918 newborns whose mothers were nonsmokers, 8,745 CpGs

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Table 2. Top 25 Significant CpGs Associated with Maternal Sustained Smoking Based on the 450 K Array[a]

CpG	Chromosome	Position	Gene Name	Effect	SE	P-Value	FDR
cg05575921	5	373378	*AHRR;AHRR*	−0.058	0.0017	2.58×10^{-262}	1.11×10^{-256}
cg25949550	7	145814306	*CNTNAP2*	−0.015	0.00048	2.96×10^{-207}	6.39×10^{-202}
cg12803068	7	45002919	*MYO1G*	0.064	0.0022	1.44×10^{-185}	2.07×10^{-180}
cg04180046	7	45002736	*MYO1G*	0.058	0.0020	4.22×10^{-179}	4.56×10^{-174}
cg09935388	1	92947588	*GFI1;GFI1;GFI1*	−0.10	0.0036	2.38×10^{-166}	2.06×10^{-161}
cg14179389	1	92947961	*GFI1;GFI1;GFI1*	−0.067	0.0026	2.23×10^{-142}	1.61×10^{-137}
cg12876356	1	92946825	*GFI1;GFI1;GFI1*	−0.088	0.0035	7.88×10^{-140}	4.86×10^{-135}
cg05549655	15	75019143	*CYP1A1*	0.035	0.0016	2.57×10^{-113}	1.39×10^{-108}
cg09662411	1	92946132	*GFI1;GFI1;GFI1*	−0.060	0.0027	1.77×10^{-112}	8.49×10^{-108}
cg11924019	15	75019283	*CYP1A1*	0.036	0.0017	3.78×10^{-104}	1.63×10^{-99}
cg18092474	15	75019302	*CYP1A1*	0.052	0.0024	4.06×10^{-103}	1.59×10^{-98}
cg22549041	15	75019251	*CYP1A1*	0.059	0.0028	2.27×10^{-99}	8.17×10^{-95}
cg12101586	15	75019203	*CYP1A1*	0.043	0.0021	9.36×10^{-90}	3.11×10^{-85}
cg18146737	1	92946700	*GFI1;GFI1;GFI1*	−0.060	0.0030	4.21×10^{-88}	1.30×10^{-83}
cg22132788	7	45002486	*MYO1G*	0.014	0.0007	5.34×10^{-88}	1.54×10^{-83}
cg22937882	5	405774	*AHRR;AHRR*	0.012	0.00059	9.38×10^{-88}	2.53×10^{-83}
cg19089201	7	45002287	*MYO1G*	0.016	0.00081	2.34×10^{-86}	5.93×10^{-82}
cg18316974	1	92947035	*GFI1;GFI1;GFI1*	−0.041	0.0021	4.04×10^{-82}	9.69×10^{-78}
cg06338710	1	92946187	*GFI1;GFI1;GFI1*	−0.040	0.0021	6.21×10^{-81}	1.41×10^{-76}
cg07339236	20	50312490	*ATP9A*	−0.013	0.00072	6.57×10^{-76}	1.42×10^{-71}
cg13570656	15	75019196	*CYP1A1*	0.040	0.0023	5.28×10^{-69}	1.09×10^{-64}
cg23576855	5	373299	*AHRR;AHRR*	−0.040	0.0023	1.41×10^{-66}	2.78×10^{-62}
cg21161138	5	399360	*AHRR;AHRR*	−0.018	0.0010	2.39×10^{-66}	4.49×10^{-62}
cg11429111	5	134813329		0.030	0.0018	6.27×10^{-63}	1.13×10^{-58}
cg23067299	5	323907	*AHRR;AHRR*	0.025	0.0015	1.11×10^{-62}	1.91×10^{-58}

[a]FDR: false discovery rate; SE: standard error.

were FDR significant (λ = 1.29), after removing 117 based on leave-one-out analyses (Table S4, Figure S1). This lambda is smaller than what has been reported in prior large smoking epigenome-wide association studies (range: 1.87−2.84).[4,46,47] Table 2 shows the results for the top 25 CpGs. Of the 8,745 CpGs, maternal smoking was associated with higher methylation at 5,745 (65.7%) CpGs (median 0.005 higher where the range of the methylation values is 0−1) and lower methylation at 3,000 (34.3%) CpGs (median −0.005 lower). In the dose−response analyses based on number of cigarettes smoked per day, there were 1,202 unexposed newborns, 408 newborns whose mother reported smoking <5 cigarettes per day, and 626 whose mother smoked 5+ cigarettes per day. Of the 8,745 CpGs significantly differentially methylated with sustained smoking, nearly all (98.6%) were at least nominally significant and had the same direction of association in dose−response analyses (Table S4). Compared to previous large smoking epigenome-wide meta-analyses in the literature,[4,29,47] we identified 304 novel genes.

Because one study reported some evidence that differential DNA methylation related to maternal smoking may differ by infant sex,[48] we also meta-analyzed the interaction term between maternal sustained smoking and infant sex, restricting to cohorts that had at least 15 males and 15 females exposed to maternal sustained smoking. Comparing 999 exposed newborns (535 males, 464 females) to 5,114 unexposed newborns (2,697 males, 2,417 females). Based on the p-values from the interaction term, there was no evidence that associations differed by infant sex (0 CpGs at FDR < 0.05, λ = 1.08, Figure S2).

Maternal Secondhand Smoke (SHS), Maternal Former Smoking, Paternal Smoking Around Conception, and Paternal Former Smoking

Among mothers classified as nonsmokers during pregnancy, no CpGs were significantly differentially methylated with maternal SHS exposure (1,514 exposed and 5,707 unexposed; λ = 1.02), maternal former smoking (1,679 exposed and 4,960 unexposed; λ = 1.05), or paternal smoking around conception (1,239 exposed and 4,620 unexposed; λ = 0.99) (Figures S3−S5). Comparing offspring of 1,017 paternal former smokers to 3,922 whose fathers were categorized as never smokers, only cg24805739 (*MED13L*) was significantly differentially methylated (λ = 1.24; β=0.002, $p = 3.2 \times 10^{-8}$) (Figure S6) and remained significant in leave-one-out analyses (Figure S7). cg24805739 was not identified as having maternal sustained smoking.

Maternal Cotinine Measurements

Cotinine measurements during pregnancy were available in six cohorts (seven data sets because DCHS conducted separate analyses for the 450 K and EPIC array). Three data sets included plasma cotinine measurements measured in the second or third trimester and four had urine measured in the first or third trimester. Cotinine was modeled continuously. All but one used the 450 K array, so meta-analyses were restricted to CpGs on the 450 K array.

In 1,035 newborns with cotinine measured in plasma, 110 CpGs were FDR significantly differentially methylated (λ = 1.05; Figure 2A). Associations with cotinine for these 110 differentially methylated CpGs were positive at 62 CpGs (56%) and negative at 48 CpGs (44%; Figure S8). For urinary cotinine, analyses of 556 newborns identified 93 CpGs with FDR < 0.05 (λ = 1.08; Figure 2B) and associations were

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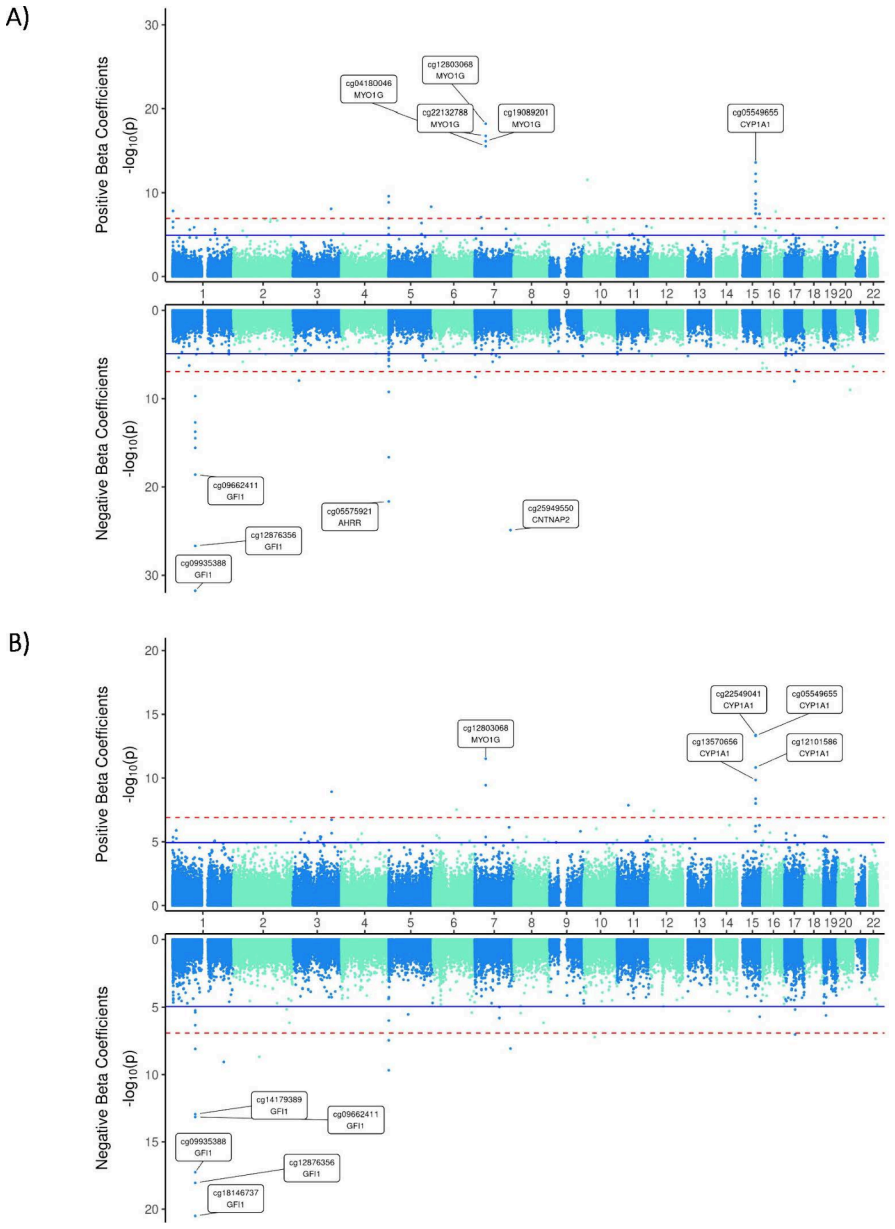

Figure 2. Miami plots of meta-analyzed results using the 450 K CpGs for maternal cotinine measured in (A) blood and (B) urine. CpGs are Bonferroni significant if they exceed the red dashed lines and/or FDR significant if they exceed the blue solid lines. The top five CpGs with a positive or negative beta coefficient and their gene annotation are noted.

positive at 64 CpGs (69%) and negative at 29 CpGs (31%) (Figure S9). Of the 110 CpGs that were FDR significant in relation to plasma cotinine, 24 (22%) were also FDR significant and 60 (54.5%) were nominally significant and had the same direction of association with urinary cotinine. Comparing these findings to the CpGs FDR significant with maternal sustained smoking in the larger analysis based on questionnaire data, there was overlap for 87 CpGs (79%) with plasma cotinine and 54 (58%) with urinary cotinine (Table S4). We identified 12 novel genes with maternal cotinine measured in blood and 29 novel genes in urine not identified in previous large publications[4,29,47] or in our sustained smoking results from this study. There was also notable overlap with FDR significant results from the dose−response analyses based on the number of cigarettes smoked per day: 86 CpGs (78%) with plasma cotinine and 47 (51%) with urinary cotinine (Table S4).

To assess whether there are differentially methylated CpGs associated with maternal SHS exposures, we restricted to newborns whose mothers were considered nonsmokers during pregnancy. Those who had cotinine levels indicative of active smoking were not included in this analysis. Of the 882 with plasma cotinine available, 436 CpGs were significantly differentially methylated (FDR < 0.05; λ = 1.13) (Figure S10), of which only 9 (2%) overlapped with the FDR significant maternal sustained smoking CpGs. Urinary maternal cotinine measurements were available for 419 newborns and 1,261 CpGs were FDR significant (λ = 1.46) (Figure S11), of which 20 (1.6%) overlapped with the maternal sustained smoking FDR significant CpGs (Table S4). Of the 436 CpGs FDR significant in plasma, only cg11081833 (*LGALS2*) overlapped with the FDR significant CpGs in urine, and 42 (9.6%) nominally overlapped.

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Functional Downstream Analyses

Functional downstream analyses were conducted for four exposures: maternal sustained smoking, paternal former smoking, and cotinine measured in the blood or urine for all newborns. Given the limited overlap of CpGs identified in the cotinine meta-analyses restricted to nonsmokers between urine and blood measures, these findings may not be robust and were not considered in downstream analyses.

Functional Enrichment Using eFORGE

The top 1000 CpGs from each exposure were consistently enriched for DNase I hotspots in several eFORGE tissue types with some of the strongest signals in fetal lung and fetal heart (Figures S12A, S13A, S14A, and S15A). For analysis across 15 chromatin states, the top 1000 CpGs for maternal sustained smoking were enriched for several different chromatin states (e.g., flanking active transcription start sites, enhancers, transcription at gene 5′ and 3′) across several tissues, including blood, lung, and digestive tissues (Figure S12B). In general, many tissues were enriched for "flanking active transcription start site" and "enhancer" categories (Figures S13B and S14B). The top 1000 CpGs related to maternal sustained smoking were also enriched for enhancers and transcription start sites in the blood, placenta, lung, and heart. There were fewer chromatin state enrichments for the top 1000 CpGs for paternal former smoker, including active transcription start sites in brain, digestive, and heart tissues (Figure S15B). The top 1000 CpGs from maternal sustained smoking, maternal cotinine measured in blood, and maternal cotinine measured in urine were consistently enriched for H3K4me1, H3K4me3, and H3K27me3 histone markers in several tissues, including blood, lung, and several fetal tissues (e.g., lung, stomach, small intestine) (Figures S12C, S13C, and S14C). There were fewer enrichments of histone marks with the top 1000 CpGs for paternal former smoker, including H3K4me1 in blood and H3K4me3 in fetal heart (Figure S15C). Taken together, these results show that different smoking-associated CpGs lists are strongly enriched for enhancers and promoters active in multiple tissues, with a slightly weaker signal for paternal former smoker.

Pathway Analyses Using MethylGSA

Of the 109 pathways evaluated by MethylGSA 41 were nominally significantly enriched in at least one of the four exposures: 17 with maternal sustained smoking, 4 with maternal cotinine measured in blood, 8 with maternal cotinine measured in urine, and 18 with paternal former smoker (Tables S5–S8, Figure 3). Five pathways overlapped with at least one other exposure, with "amyotrophic lateral sclerosis" being enriched in the greatest number of exposures: maternal sustained smoking, maternal blood cotinine, and paternal former smoking.

Enrichment of Traits in EWAS Atlas

The top 1000 CpGs across the four exposures were enriched for 156 different traits previously studied with EWAS: 108 with maternal sustained smoking, 98 with cotinine measured in blood, 76 with cotinine measured in urine, and 44 with paternal former smoking (Table S9). Sixty-one traits were enriched across all three maternal smoking exposures, and 14 of them were also enriched with paternal former smoking.

Cis-eQTM

For maternal sustained smoking, of the 8,745 FDR significant CpGs, 7,899 were available in the HELIX data set of 832 children and 16% were associated with nearby gene expression at FDR < 0.05 (Table S10). Of the 110 FDR significant CpGs associated with cotinine measured in blood, 100 were available in HELIX and 24% were associated with *cis*-gene expression (Table S11). Of the 93 CpGs associated with urinary cotinine, 87 were available in HELIX and 11.5% were associated with *cis*-gene expression (Table S12). The one CpG (cg24805739) identified with paternal former smoking was not associated with nearby gene expression (Table S13).

More *cis*-eQTMs were observed in the larger BIOS Consortium of 3,075 adults. For maternal sustained smoking, 8,192 CpGs were available, and 3,741 (45.7%) were associated with gene expression at FDR < 0.05 (Table S14). Of the 110 CpGs associated with maternal cotinine measured in blood, 103 CpGs were available in BIOS, and 65 (63.1%) were associated with nearby gene expression (Table S15). For maternal urinary cotinine, 86 CpGs were available in BIOS, and 39 (45.3%) were associated with nearby gene expression (Table S16). The CpG with paternal former smoking was not associated with gene expression (Table S17).

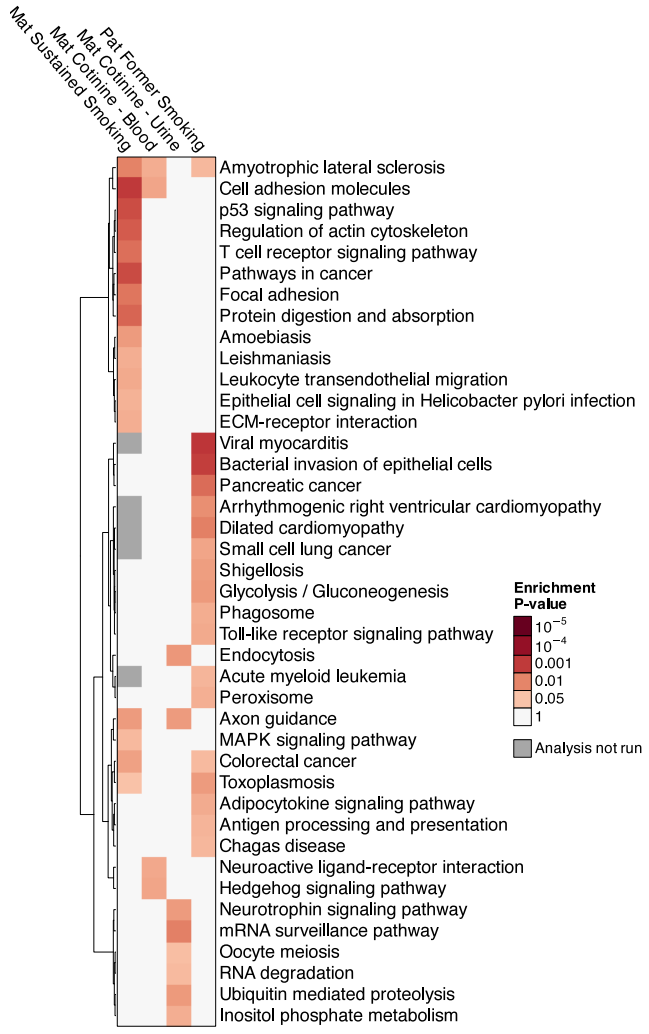

Figure 3. Heatmap of enriched pathways for maternal sustained smoking, maternal cotinine measured in blood, maternal cotinine measured in urine, and paternal former smoker, based on 450 K CpGs. The color of the cell reflects the enrichment p-value, where white indicates no association and the darker shade of red indicates more significance. Gray cells indicate the pathway was not analyzed for that exposure.

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Table 3. FDR Significant CpGs Unique to the EPIC Array Associated with Maternal Secondhand Smoke or Paternal Former Smoking (i.e., Quit before Pregnancy) in Newborns of Nonsmoking Mothers[a]

CpG	Chromosome	Position	Gene Name	Effect	SE	P-value	FDR	Direction
Maternal SHS (n = 2,155)								
cg09745820	1	75938229	*SLC44A5*	0.003	0.0006	3.51×10^{-09}	0.001	−++[b]
cg08969523	6	51355860		0.004	0.0007	1.37×10^{-08}	0.002	+−+[b]
cg26868111	1	117080375	*CD58*	0.004	0.0007	2.26×10^{-08}	0.003	−++[b]
cg27231550	1	70503881	*LRRC7*	0.003	0.0006	3.38×10^{-08}	0.003	−++[b]
cg07211787	9	29349163		0.010	0.002	1.77×10^{-07}	0.01	+++[b]
Paternal Former Smoking (n = 2,056)								
cg05366393	6	109703123	*CD164*	−0.0005	8.90×10^{-05}	5.88×10^{-08}	0.01	+−[c]
cg01634622	7	156174435		0.002	0.0003	6.27×10^{-08}	0.01	+++[c]
cg26340487	3	33479298	*UBP1*	0.004	0.0007	1.13×10^{-07}	0.01	+++[c]
cg06957099	11	665386	*DEAF1*	0.002	0.0003	2.36×10^{-07}	0.02	+++[c]

[a]FDR: false discovery rate; SE: standard error. [b]Direction of effect for Upstate KIDS, CCLS4 HP, and MoBa-START, respectively. [c]Direction of effect for CCLS4 EU, CCLS4 HP, and MoBa-START, respectively.

Meta-Analyses of CpGs Unique to EPIC Array

Comparing 206 newborns exposed to maternal sustained smoking to 1,230 unexposed, differential DNA methylation at FDR < 0.05 was seen for 211 CpGs unique to the EPIC array (λ = 1.29; Figures S16 and S17, Table S18). The 211 CpGs annotated to 125 unique genes, of which nine were not identified in our 450 K meta-analysis or previous smoking meta-analyses.[4,29,47] Among mothers who reported to be nonsmokers, no associations were observed with maternal former smoker (703 exposed and 1,841 unexposed; λ = 1.05; Figure S18) or paternal smoking around conception (280 exposed and 1,265 unexposed; λ = 1.05; Figure S19). Comparing 295 newborns whose mothers were exposed to SHS to 1,860 unexposed, five CpGs had FDR < 0.05 (λ = 1.05; Table 3; Figures S20 and S21). Paternal former smoking (400 exposed versus 1,656 never smoker) was associated with DNA methylation at four CpGs at FDR significance (λ = 1.14; Table 3; Figures S22 and S23). The 5 CpGs with maternal SHS and 4 CpGs with paternal former smoking were not nominally significant in maternal sustained smoking.

DISCUSSION

In this large multicohort meta-analysis of 11,175 parent-newborn pairs, we examined the impact of five questionnaire-based prenatal tobacco smoking exposures (i.e., maternal sustained smoking, maternal SHS maternal former smoker, paternal smoking around conception, and paternal former smoker). The large number of significant CpGs with maternal sustained smoking compared to other prenatal smoking exposures strongly suggests that the intrauterine environment has the greatest impact on the newborn methylome. We also identified some novel smoking-related genes in this study from the 450 K array for exposures based on questionnaire, for cotinine measured in blood or urine, and for CpGs unique to the EPIC array. Interestingly, although few signals were observed, we identified CpGs associated with paternal former smoking and maternal SHS, and none associated with paternal smoking around conception.

While some methylation signatures related to maternal smoking during pregnancy overlap with personal smoking CpGs in adults, there are also signals unique to newborns. The PACE consortium has compared their previous meta-analysis of maternal sustained smoking in relation to newborn DNA methylation to methylation signatures of personal smoking in adults in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium.[29,49] We reported that 69% of maternal sustained smoking CpGs were unique to newborns (i.e., not identified in adults in relation to their own smoking).[49] Unlike in the prior PACE meta-analysis, in the current study, we were able to examine maternal sustained smoking using biomarkers in a subset of studies (i.e., cotinine measured in urine or blood) along with questionnaire data in the larger data set. This enabled us to confirm that most maternal smoking related CpGs can be robustly identified across the three exposure measurement approaches. Despite the much smaller sample sizes with either cotinine measurements compared to questionnaire data, we identified 41 novel genes with maternal cotinine measured in blood or in urine not previously identified in this or other large epigenome-wide meta-analyses of questionnaire-based smoking exposure, including adults.[4,29,47] Looking up these 41 novel genes in an EWAS of cotinine in 500 adults,[50] none overlapped, suggesting that they may be unique to newborns. Downstream analyses further demonstrated that results from each model implicated some different traits, pathways, and functional analyses, suggesting that different insights might be gleaned based on exposure matrix.

The PACE Consortium previously reported that differential DNA methylation was more pronounced in women who continued smoking throughout pregnancy than the approximately half of women who quit smoking early in pregnancy.[4] Furthermore, in a smaller study focusing on 26 top maternal sustained smoking-related CpGs, there was evidence that newborns of mothers who quit before pregnancy or who smoked at the beginning of pregnancy but quit by the 18th week of pregnancy had methylation levels similar to newborns of mothers who never smoked.[51] In the current study, we wanted to expand the literature by conducting epigenome-wide analyses to determine whether there may be other CpGs that may be impacted by maternal former smoking. This study did not provide evidence of differential methylation in newborns related to maternal former smoking before pregnancy.

Despite the known effects of maternal SHS during pregnancy,[2] this exposure has not been well-studied in relation to newborn methylation. One study investigated paternal smoking during pregnancy as a proxy for maternal SHS exposure (n = 233 exposed) and did not report any significant associations with CpGs on the 450 K array.[52] While our much larger study (n = 1,514 exposed) confirms their findings, we also reported 5 CpGs unique to the EPIC array in 295 exposed

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that need to be confirmed. In another study, the authors identified over 29,000 significant CpGs in 79 women with plasma cotinine in the SHS exposure range.[52] Of the 27,081 CpGs that overlapped in our larger meta-analyses of maternal cotinine in the SHS range in plasma (N = 882) or urine (N = 419), only 98 (0.4%) were FDR significant in at least one of our models. Exposure to SHS is more challenging to assess by questionnaire than with personal smoking. In our meta-analysis, SHS was additionally challenging to operationalize because each cohort collected different information. For example, some cohorts accessed SHS based on partner's smoking status only, and others collected detailed information about SHS outside the home. We tried to objectively capture SHS exposure in the subset of the data with cotinine. However, each cohort used a different assay, some of which are not sufficiently sensitive at the low levels required to detect passive exposure. Furthermore, cotinine has a half-life of ∼16 h in plasma and 17−19 h in urine,[53] and thus reflects only short-term exposure rather than exposure across pregnancy. Cotinine was not measured at the same time point in pregnancy across studies, potentially contributing to differences by matrix. In addition, given the weak associations expected for SHS, the limited sample size with 882 mother-child pairs with plasma cotinine and 419 with urinary cotinine when restricting to levels not indicative of active smoking may lead to less reliable results. We did find that there was more overlap between urine and blood results when examining levels indicative of active smoking. Our results suggest that robust differential methylation signatures related to maternal SHS will be challenging to discover in newborns given limitations in assessing SHS exposure.

In addition to exposing the mother to SHS, paternal smoking can affect the sperm epigenome,[54] which may influence offspring health outcomes.[55,56] One study reported that DNA methylation at 33 CpGs was related to paternal smoking (132 exposed) in offspring aged 11−54 years.[57] A larger study (328 exposed) reported differential methylation at 2 CpGs in offspring aged 7−50 years.[58] We were able to look up 34 of those CpGs in our paternal current smoking study, but none were nominally significant. Our large meta-analysis of paternal smoking around conception did not identify any significant CpGs, which is consistent with what is known about epigenetic marks after fertilization, in which the zygote's epigenome mostly demethylates and new methylation marks are established.[59]

Interestingly, our study identified some differentially methylated CpGs in newborns related to having fathers who were former smokers. There is some evidence that prepuberty exposures in men could affect offspring health[60] and methylome,[58] but because not all studies collected information on age when father start and stopped smoking, we were unable to conduct a meta-analysis restricting to fathers who smoked before puberty. Of the 19 CpGs reported to be associated with paternal smoking onset <15 year in offspring ages 7 to 50 years,[58] 17 were available in our study and none were nominally significant. cg24805739 identified among our 450 K CpGs annotates to *MED13L*, which has been linked to the development of congenital heart defects and developmental delay and intellectual disability with associated facial dysmorphism.[61] Interestingly, the top 1000 CpGs for paternal former smoking were most significantly enriched for DNase I hotspots in the fetal brain and heart.

Our study had some limitations. Like early genome-wide association studies of genetic variation, most of our study population was not diverse, in terms of genetic ancestry. Furthermore, most studies in PACE measured DNA methylation using the 450 K array in the blood. Prenatal smoking-related DNA methylation signatures in blood may not reflect patterns in other tissues, as has been observed with placenta tissue.[46] In our maternal urinary cotinine analyses, we were unable to account for variations in urinary dilutions, as only one study (INMA) measured creatinine. However, in a sensitivity analysis, including creatinine in the model did not appreciably change the results (Table S19). In an ideal study, the unexposed group for maternal SHS and paternal smoking would be restricted to women who never smoked and were never exposed to SHS. However, information about SHS prior to pregnancy was not collected routinely. Further, given that about half of women smoking early in pregnancy quit (most likely due to the strong public health messaging around adverse impacts of tobacco smoke on the fetus),[4] nonsmoking women uniquely exposed to SHS during pregnancy are likely to be uncommon. Because we did not observe any significant findings with maternal former smoking, we do not expect our findings to appreciably affect the unexposed groups we used.

In our large meta-analysis, we examined a comprehensive range of prenatal tobacco smoking exposures that have not been well-studied in relation to newborn DNA methylation, including objective measurements of maternal smoking during pregnancy. Our study did not identify substantial DNA methylation signatures of SHS in pregnancy, maternal smoking before pregnancy, or paternal smoking before and around conception. Methylation signatures of maternal sustained smoking related CpGs did not differ by infant sex. Finally, similar but nonoverlapping patterns of DNA methylation were observed between questionnaire-based maternal sustained smoking, maternal cotinine measured in blood, and maternal cotinine measured in urine. Using the newer EPIC array, we identified some novel genes related to maternal sustained smoking that had not been previously reported. These findings shed light on potential mechanisms underlying the impacts of various forms of prenatal exposure to smoking on offspring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/EHP.6c00142?goto=supporting-info.

Supporting tables (XLSX)
Supporting methods and figures (DOCX)

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Notes

The authors declare no competing financial interest.

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