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Genome-wide DNA methylation study in human placenta identifies novel loci associated with maternal smoking during pregnancy

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Abstract

Background: We conducted an epigenome-wide association study (EWAS) of DNA methylation in placenta in relation to maternal tobacco smoking during pregnancy and examined whether smoking-induced changes lead to low birthweight.

Methods: DNA methylation in placenta was measured using the Illumina HumanMethylation450 BeadChip in 179 participants from the INfancia y Medio Ambiente (INMA) birth cohort. Methylation levels across 431 311 CpGs were tested for

differential methylation between smokers and non-smokers in pregnancy. We took forward three top-ranking loci for further validation and replication by bisulfite pyrosequencing using data of 248 additional participants of the INMA cohort. We examined the association of methylation at smoking-associated loci with birthweight by applying a mediation analysis and a two-sample Mendelian randomization approach.

Results: Fifty CpGs were differentially methylated in placenta between smokers and non-smokers during pregnancy [false discovery rate (FDR) < 0.05]. We validated and replicated differential methylation at three top-ranking loci: cg27402634 located between *LINC00086* and *LEKR1*, a gene previously related to birthweight in genome-wide association studies; cg20340720 (*WBP1L*); and cg25585967 and cg12294026 (*TRIO*). Dose–response relationships with maternal urine cotinine concentration during pregnancy were confirmed. Differential methylation at cg27402634 explained up to 36% of the lower birthweight in the offspring of smokers (Sobel *P*-value < 0.05). A two-sample Mendelian randomization analysis provided evidence that decreases in methylation levels at cg27402634 lead to decreases in birthweight.

Conclusions: We identified novel loci differentially methylated in placenta in relation to maternal smoking during pregnancy. Adverse effects of maternal smoking on birthweight of the offspring may be mediated by alterations in the placental methylome.

Key words: birthweight, DNA methylation, epigenetics, fetal programming, placenta, tobacco smoking

Key Messages

- Maternal tobacco smoking during pregnancy is associated with genome-wide DNA methylation changes in human placenta.
- Differential methylation at cg27402634, located between *LINC00086* and *LEKR1*, mediates in part the association between maternal smoking during pregnancy and low birthweight of the offspring.
- A two-sample Mendelian randomization analysis provides evidence that decreases in placental methylation levels at cg27402634 lead to decreases in birthweight.
- Enrichment analysis reveals functional pathways related to signalling by growth factors, inflammation, oxidative stress, peroxisomal metabolism, and myometrial relaxation and contraction.
- DNA methylation changes in placenta associated with maternal tobacco smoking differ largely from those identified in cord blood and may be functionally relevant for placental and fetal development.

Introduction

Tobacco smoking is still common among pregnant women despite the increased awareness of adverse health consequences for the offspring, including fetal growth restriction, preterm birth, low birthweight, and higher risk of respiratory and cardiometabolic diseases later in life.[1–3] Early-life exposures may have long-lasting effects on development, metabolism and health via epigenetic phenomena, including DNA methylation changes.[4] DNA methylation is the reversible and mitotically heritable attachment of a methyl group to a nucleotide that could influence gene expression and downstream phenotypes.

Several studies have used cord blood or whole blood to provide good insight into the effects of maternal smoking in pregnancy on DNA methylation signatures in the offspring.[5–12] Two studies showed an association between maternal smoking during pregnancy and methylation at differentially methylated regions regulating the imprinted Insulin-like Growth Factor 2 (*IGF2*) gene in cord blood DNA.[5,6] Furthermore, epigenome-wide association study (EWAS) analyses using the Infinium HumanMethylation450 BeadChip (450K) have identified differential DNA methylation in cord blood in genes related to the detoxification of the components of tobacco smoke, developmental processes, nicotine dependence, smoking cessation, placental and embryonic development, cell growth, and regulation of hormone secretion and natriuresis.[7–12] Moreover, several studies have revealed

lasting effects of *in utero* exposure to tobacco smoking on offspring DNA methylation features (i.e. *AHRR*, *MYO1G*, *CYP1A1* and *CNTNAP2*) that persist through childhood and adolescence.[8,11,13–15]

Since DNA methylation profiles are tissue-specific to a large extent, elucidating the tissue-specific epigenetic variation in response to prenatal exposures is crucial to understand mechanisms leading to health effects. Placental methylome may act as a functional record of *in utero* exposures; however, only a few small studies have assessed the effect of maternal smoking during pregnancy on DNA methylation patterns in human placenta[16–19]—an organ that plays a key role in controlling fetal growth and development. A candidate-gene-based study showed that maternal smoking increased *CYP1A1* expression levels in placenta through DNA methylation changes.[16] Using the Illumina 27K chip, Suter *et al.* reported altered placental methylation and expression patterns of genes encoding molecules involved in hypoxia response and oxidative stress regulating pathways in relation to maternal tobacco smoking, showing that some of the CpG sites were associated with low birthweight in the offspring.[17] Using the same methylation platform, Maccani *et al.* reported that placental methylation of a number of probes within *RUNX3* was associated with smoking during pregnancy and one of them was related to decreased gestational age.[18] Lastly, an EWAS investigating DNA methylation features using the Illumina 450K platform in placental tissue collected at 85–90 days post conception reported hypomethylation of probes in *GTF2H2C* and *GTF2H2D* loci in relation to nicotine exposure in pregnancy and demonstrated enrichment of gene-sets associated with asthma and immune disorders.[19]

Here, we conducted an EWAS analysis on placental DNA methylation in relation to maternal tobacco smoking during pregnancy using the Illumina 450K platform. In a second step, using bisulfite pyrosequencing, we validated the results of three top-ranking novel loci showing differential methylation in smokers and replicated the findings in an independent study sample. We additionally examined whether placental DNA methylation at identified loci mediates the association of maternal smoking with the birthweight of the offspring by applying a mediation analysis and a two-sample Mendelian randomization approach.

Methods

Study participants

The present study used data from participants recruited between 2003 and 2008 in the four *de novo* cohorts sited in Asturias, Gipuzkoa, Sabadell and Valencia of the INfancia y Medio Ambiente (INMA) Project, a population-based mother–child cohort study in Spain.[20] Overall, 2506 mother–child pairs were followed until birth and placental DNA samples were available for 450 of them. In order to maximize the use of the genome-wide methylation data, a sub-sample of 179 mother–child pairs with complete datasets in terms of prenatal exposures and health outcomes formed our discovery population. For our replication study, we used data of 248 additional mother–child pairs of the INMA cohort (Supplementary Figure 1, available as Supplementary data at *IJE* online). Comparison with eligible participants of the INMA cohort is shown in Supplementary Table 1 (available as Supplementary data at *IJE* online). The study was approved by the ethical committees of the centres involved in the study and written informed consent was obtained from all participants.

Maternal tobacco smoke exposure during pregnancy

Active maternal smoking during pregnancy was assessed through questionnaires administered face to face by trained interviewers in weeks 12 and 32 of pregnancy. The main exposure variable was the classification of maternal tobacco-smoking status: 'non-smokers during pregnancy' were defined as women reporting no active tobacco smoking at 12 and 32 weeks of pregnancy, and 'smokers during pregnancy' were those reporting tobacco smoking at both 12 and 32 weeks of pregnancy. Maternal urine cotinine concentration was determined with a competitive enzyme immunoassay in urine samples collected during the third trimester of pregnancy. Sensitivity (0.96) and specificity (0.95) for the cut-off point of 50 ng/ml showed good agreement between self-reported smoking and urine cotinine concentration.[21] For further analyses, maternal urine cotinine concentration was cut off in three categories: <50 ng/ml as non-active smoking, 50–2000 ng/ml and >2000 ng/ml.

EWAS analysis in placenta

Collected placentas were stored at −80°C until processing. Biopsies of approximately 5 cm^3 were obtained from the inner region of the placenta and genomic DNA was isolated using the DNeasy® Blood and Tissue Kit (Qiagen, CA, USA) (see Supplementary material for additional details, available as Supplementary data at *IJE* online).

Genome-wide DNA methylation examination was performed using the Infinium Human-Methylation450 BeadChip (Illumina, San Diego, CA, USA) following the manufacturer's recommendations. BeadChips were scanned with an Illumina iScan and image data were uploaded into the Methylation Module of Illumina's analysis software GenomeStudio (lllumina, SanDiego, CA USA)

and converted in β-values that range from 0 (unmethylated) to 1 (fully methylated) and represent the fraction of methylation at a given CpG site. After data quality control and normalization processes (see Supplementary material for details, available as Supplementary data at *IJE* online), 433 131 probes in 179 samples remained for further analyses.

In the discovery study, multivariable robust linear regression was employed using the R package, MASS, to test the association between the normalized β-value at each CpG as the dependent variable and maternal tobacco smoking during pregnancy (yes vs no) as the independent variable. Covariates were selected among a list of potential a priori confounders including area of study, child's sex, gestational age, maternal age, maternal social class based on occupation, parity, father's tobacco-smoking habits and potential batch effects (i.e. chip and PCR plate). Information on maternal occupation during pregnancy (based on the Spanish adaptation of the international ISCO88 coding system), maternal age, parity and fathers' tobacco-smoking habits was obtained through questionnaires. Offspring's sex was obtained from clinical records and gestational age was calculated by ultrasounds. Covariates included in the final model were selected by testing the difference of the correlation of *P*-values before and after adjustment for each variable using a Kolmogorov–Smirnov test. False discovery rate (FDR)-corrected *P*-values were determined according to the method of Benjamini and Hochberg.[22] Further details can be found in the Supplementary material, available as Supplementary data at *IJE* online.

Validation and replication study of top-ranking loci

We performed validation and replication analyses for four maternal-smoking-associated CpG sites located in the three top-ranking genes based on the results of our EWAS analysis and considering the magnitude of the observed methylation difference: cg27402634 located between *LINC00086* and *LEKR1* genes; cg20340720 at *WBP1L*; and cg12294026 and cg25585967 at *TRIO*. Pyrosequencing assays were designed using the PyroMark Q96 ID pyrosequencing system (Qiagen, Germany). Bisulfite-DNA amplification and sequencing was performed in duplicate using the primers and assay conditions in Supplementary Table 2 (available as Supplementary data at *IJE* online). Fully methylated and fully unmethylated control samples were included in all experiments. Adjusted mixed linear regression models were run including repeated pyrosequencing measurements as random intercept. Here, individual random effects were specified to take into account the correlation between replicates from the same individual and the information of the working PCR plate. All statistical analyses were performed using the R statistical package (R Foundation for Statistical Computing, Vienna, Austria).

Annotation and enrichment and networks analyses

After annotation of top-ranking differentially methylated CpG sites, gene-set enrichment analyses were performed with the ConsensusPathDB programme.[23] Functional networks were investigated with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING)[24] (see Supplementary material for further details, available as Supplementary data at *IJE* online).

Mediation analysis

We tested the role of methylation changes at the top-ranking CpGs validated by pyrosequencing assay in mediating the association between maternal smoking during pregnancy and birthweight of the offspring, recorded by trainee midwifes at delivery, using the method of Baron and Kenny[25] and the Sobel test.[26]

Causal inference approaches

Two approaches were applied to infer causality: (i) considering paternal smoking as a negative control exposure for the association between maternal smoking and DNA methylation in placenta[27] and (ii) a two-sample Mendelian randomization (MR) approach to evaluate the causal effect of placenta DNA methylation changes on birthweight.[28] Maternal urine cotinine levels in non-smokers during pregnancy did not differ according to paternal smoking (mean 7.8 ng/ml among women of non-smokers vs 11.8 ng/ml among women of smokers), which indicates that paternal smoking can be used as a negative control exposure. For a two-sample MR analysis, first, we looked for methylation quantitative trait locus (meQTLs) for the top-ranked CpG site (cg27402634) in the present study and then we analysed their association with birthweight *z*-score in an independent dataset from the Early Growth Genetics (EGG) consortium (http://egg-consortium.org/birth-weight.html). A Mendelian randomization analysis was performed using a likelihood test for summarized data of uncorrelated genetic variants and samples[29] including MR-Egger regression to assess potential pleiotrophy[30] (see more details in the Supplementary material, available as Supplementary data at *IJE* online).

Results

Characteristics of the discovery and replication study populations are shown in Table 1. Smoking mothers more often had lower social class and fathers of children of smoking mothers more often smoked. Offspring of smoking mothers included in the discovery sample had lower birthweight, although the difference did not reach statistical significance in the replication sample.

EWAS results from the robust linear regression for 431 311 CpGs across the genome in 179 placental samples are shown in the Supplementary Figure 2 (available as Supplementary data at *IJE* online). This revealed 50 CpGs, representing 46 loci, differentially methylated in smokers compared with non-smokers during pregnancy (FDR < 0.05) (Supplementary Table 3, available as Supplementary data at *IJE* online). Among these 50 CpGs, 14 showed a difference in methylation >5%, of which 8 were hypomethylated and 6 were hypermethylated in smokers in comparison to non-smokers (Table 2).

We took forward four probes at the three top-ranking loci for validation and replication by bisulfite pyrosequencing. All of them showed effect estimates for maternal smoking consistent across the discovery and the replication study populations (Table 3). Methylation levels at cg27402634 (located between *LINC00086* and *LEKR1*) and cg20340720 (*WBP1L*) decreased in smokers during pregnancy by 9.3% and 8.7%, respectively (P-value $< 2 \times 10^{-16}$). Methylation levels at cg25585967 and cg12294026 (*TRIO*) increased among smokers in pregnancy by 6.8% and 7.4%, respectively (P-value $< 2 \times 10^{-16}$). Consistently, we found a tendency of cg27402634 and cg20340720 methylation to decrease with increasing levels of maternal urine cotinine in pregnancy and a tendency of cg25585967 and cg12294026 methylation to increase with increasing levels of maternal urine cotinine (Figure 1).

Associations between maternal smoking and DNA methylation changes at top-ranked CpGs remained essentially the same after further adjustment for paternal smoking (Supplementary Table 4, available as Supplementary data at *IJE* online), whereas estimates for paternal smoking were generally weaker than those for maternal smoking and were not robustly associated with DNA methylation changes, except for cg20340720.

Next, we tested the top-ranking CpGs for mediation in the association between maternal smoking during pregnancy and birthweight of the offspring. Both cg27402634

Table 1. Characteristics of children unexposed and exposed to maternal smoking during pregnancy in study populations

	Discovery sample ($n = 179$)			Replication sample ($n = 248$)		
	Unexposed[a] ($n = 151$)	Exposed[b] ($n = 28$)	P-value[c]	Unexposed[a] ($n = 208$)	Exposed[b] ($n = 40$)	p-value[c]
Area of study						
Valencia	17 (11.3)	3 (10.7)	0.672	39 (18.8)	13 (32.5)	0.182
Sabadell	54 (35.8)	12 (42.9)		76 (36.5)	13 (32.5)	
Gipuzkoa	55 (36.4)	7 (25.0)		86 (41.3)	14 (35.0)	
Asturias	25 (16.6)	6 (21.4)		7 (3.4)	0 (0.0)	
Maternal age (years)	31.1 (3.9)	30.8 (4.4)	0.691	30.9 (4.0)	30.3 (4.2)	0.442
Maternal social class						
I–II (Managers//technicians)	40 (26.5)	4 (14.3)	0.249	56 (26.9)	3 (7.5)	0.004
III (Skilled)	48 (31.8)	8 (28.6)		56 (26.9)	8 (20.0)	
IV–V (Semi-skilled/unskilled)	63 (41.7)	16 (57.1)		96 (46.2)	29 (72.5)	
Parity						
0	91 (60.3)	14 (50.0)	0.421	112 (53.8)	26 (65.0)	0.259
≥1	60 (39.7)	14 (50.0)		96 (46.2)	14 (35.0)	
Maternal urine cotinine in pregnancy, ng/ml	2.8 (2.8, 11.0)	1828.9 (946.9, 3020.2)	<0.001[d]	4.5 (2.8, 12.8)	2055.4 (1014.2, 3103.5)	<0.001[d]
<50 ng/ml	136 (97.8)	2 (8.0)	<0.001	187 (96.4)	0 (0.0)	<0.001
50–2000 ng/ml	3 (2.2)	11 (44.0)		6 (3.1)	18 (48.6)	
>2000 ng/ml	0.0	12 (48.0)		1 (0.5)	19 (51.4)	
Father smoking (yes)	33 (21.9)	17 (63.0)	<0.001	68 (32.7)	25 (62.5)	<0.001
Child sex (male)	74 (49.0)	19 (67.9)	0.104	109 (52.4)	19 (47.5)	0.692
Gestational age (weeks)	39.6 (1.3)	39.8 (1.2)	0.534	39.7 (1.3)	39.8 (1.2)	0.465
Birthweight (g)	3344 (408)	3100 (356)	0.003	3271 (456)	3185 (424)	0.282

Data shown as % or mean ± (SD). Except for maternal urine cotinine: median (interquartile range, p25–p75).

[a]Women reporting no tobacco smoking at 12 and 32 weeks of pregnancy. [b]Women reporting tobacco smoking at 12 and 32 weeks of pregnancy ('sustained smoking'). [c]Otherwise indicated, P-values are given for independent samples t-test (continuous) or chi-square test (categorical). [d]p-value for Kruskal-Wallis test.

Table 2. Top 14 loci with methylation difference > 5% at FDR < 0.05 in placenta in relation to maternal smoking during pregnancy, sorted by *P*-value

CpG site	Chr: position[a]	Gene or nearest genes at 3' and 5'	Diff.[b]	*p*-value	*q*-value[c]	Mean (SE) methylation (%)	
						Non-smokers	Smokers
cg27402634	3: 156536860	*LINC00086;LEKR1*	−16.7	2.70×10^{-28}	1.20×10^{-22}	79.3 (1.0)	62.6 (1.2)
cg20340720	10: 104512524	*WBP1L*	−6.7	1.80×10^{-11}	3.90×10^{-06}	60.4 (0.9)	53.6 (1.0)
cg25585967	5: 14452105	*TRIO*	5.9	5.50×10^{-10}	7.90×10^{-05}	70.9 (0.8)	76.8 (0.1)
cg26843110	15: 74935742	*EDC3*	−5.9	1.10×10^{-09}	1.20×10^{-04}	67.4 (0.7)	61.6 (0.1)
cg17823829	1: 202765755	*KDM5B*	7.7	3.80×10^{-09}	2.70×10^{-04}	68.2 (1.2)	75.8 (1.4)
cg12291408	7: 100037572	*PPP1R35;C7orf61*	−6.1	1.30×10^{-08}	7.80×10^{-04}	53.8 (1.0)	47.7 (1.1)
cg25589945	2: 46429384	*PRKCE;EPAS1*	−5.3	1.60×10^{-07}	4.70×10^{-03}	62.9 (0.8)	57.6 (1.0)
cg14044375	2: 232085909	*ARMC9*	−5.4	1.60×10^{-07}	4.70×10^{-03}	64.5 (0.9)	59.1 (1.1)
cg03978169	6: 33091358	*HLA-DPB2*	−8.3	4.50×10^{-07}	9.80×10^{-03}	55.7 (1.7)	47.4 (1.9)
cg15205441	10: 126782358	*CTBP2*	6.4	7.90×10^{-07}	0.016	47.9 (1.2)	54.4 (1.4)
cg10090414	8: 142309653	*SLC45A4;LINC01300*	6.9	1.50×10^{-06}	0.027	33.2 (1.5)	40.1 (1.7)
cg26223797	5: 54131695	*LOC102467080;ESM1*	6.2	1.70×10^{-06}	0.028	49.9 (1.2)	56.0 (1.4)
cg08692423	21: 45148865	*PDXK*	5.6	2.50×10^{-06}	0.037	22.8 (1.0)	28.4 (1.2)
cg16044379	11: 13980927	*FAR1;SPON1*	−7.2	5.20×10^{-06}	0.049	79.0 (1.5)	71.8 (1.8)

[a]Chromosome and position based on USCC Genome Browser (hg19); [b]difference in the Illumina beta-value between smokers and non-smokers expressed as percentage; [c]false discovery rate (FDR). All models adjusted for area of study, child's sex, maternal age, maternal social class, parity, chip and plate.

Table 3. Placental DNA methylation difference[a] (%) in smokers vs non-smokers during pregnancy at top-ranking CpGs obtained using bisulfite pyrosequencing

	Discovery sample		Replication sample		Pooled	
CpG	Diff. (SE)	*p*-value	Diff. (SE)	*p*-value	Diff. (SE)	*p*-value
cg27402634	−10.0 (1.8)	9.22×10^{-08}	−13.9 (1.4)	$<2 \times 10^{-16}$	−9.3 (1.0)	$<2 \times 10^{-16}$
cg20340720	−6.6 (1.2)	9.62×10^{-08}	−11.9 (1.1)	$<2 \times 10^{-16}$	−8.7 (0.7)	$<2 \times 10^{-16}$
cg25585967	7.6 (1.0)	2.35×10^{-12}	6.0 (0.7)	4.44×10^{-16}	6.8 (0.6)	$<2 \times 10^{-16}$
cg12294026	7.5 (1.3)	4.39×10^{-08}	5.5 (0.7)	9.77×10^{-15}	7.4 (0.7)	$<2 \times 10^{-16}$

[a]Estimated using mixed linear regression models adjusted for area of study, child's sex, maternal age, maternal social class, parity and plate.

(between *LINC00086* and *LEKR1*) and cg25585967 (*TRIO)* showed mediation with Sobel test *P*-value < 0.05 in the discovery sample, whereas the other two tested CpGs did not (Table 4). Differential methylation at cg27402634 and cg25585967 explained 36.5% and 5.1% of the 314-g lower birthweight in offspring of smoking mothers, respectively. Although attenuated, similar results were observed using methylation data obtained from bisulfite pyrosequencing assays (Supplementary Table 5, available as Supplementary data at *IJE* online). However, these results could not be confirmed in the replication sample.

Five meQTLs for cg27402634 were identified in placenta. Two of them were associated with birthweight (*P*-value < 0.05) according to summarized GWAS data from the EGG consortium (Supplementary Table 6, available as Supplementary data at *IJE* online). The effect of DNA methylation levels at cg27402634 on birthweight was estimated using a full set of five tag SNPs and the two closest tag SNPs surrounding cg27402634. We found that the causal estimate for 1% increase in methylation at cg27402634 on birthweight *z*-score was a 0.007 increase [95% confidence interval (CI): 0.002, 0.011] (Table 5 and Supplementary Figure 3, available as Supplementary data at *IJE* online). Moreover, since there was some heterogeneity in the effects of the SNPs on birthweight and on DNA methylation (Supplementary Figure 3, available as Supplementary data at *IJE* online), we performed MR-Egger regression. The intercept of MR-Egger regression was practically null [intercept (95% CI): –0.006 (–0.093; 0.080)] (Table 5), suggesting that, if any, there was balanced pleiotrophy. The estimate of MR-Egger regression was 0.006 (–0.016; 0.028) and, although with broader CI, it was similar to the causal estimate calculated using the LHR test (Table 5).

Genes annotated either upstream, downstream or at CpGs showing a FDR < 0.05 were enriched for pathways related to signalling by growth factors (EGFR, ERBB2, FGFR, PDGF and NGF), inflammation and oxidative

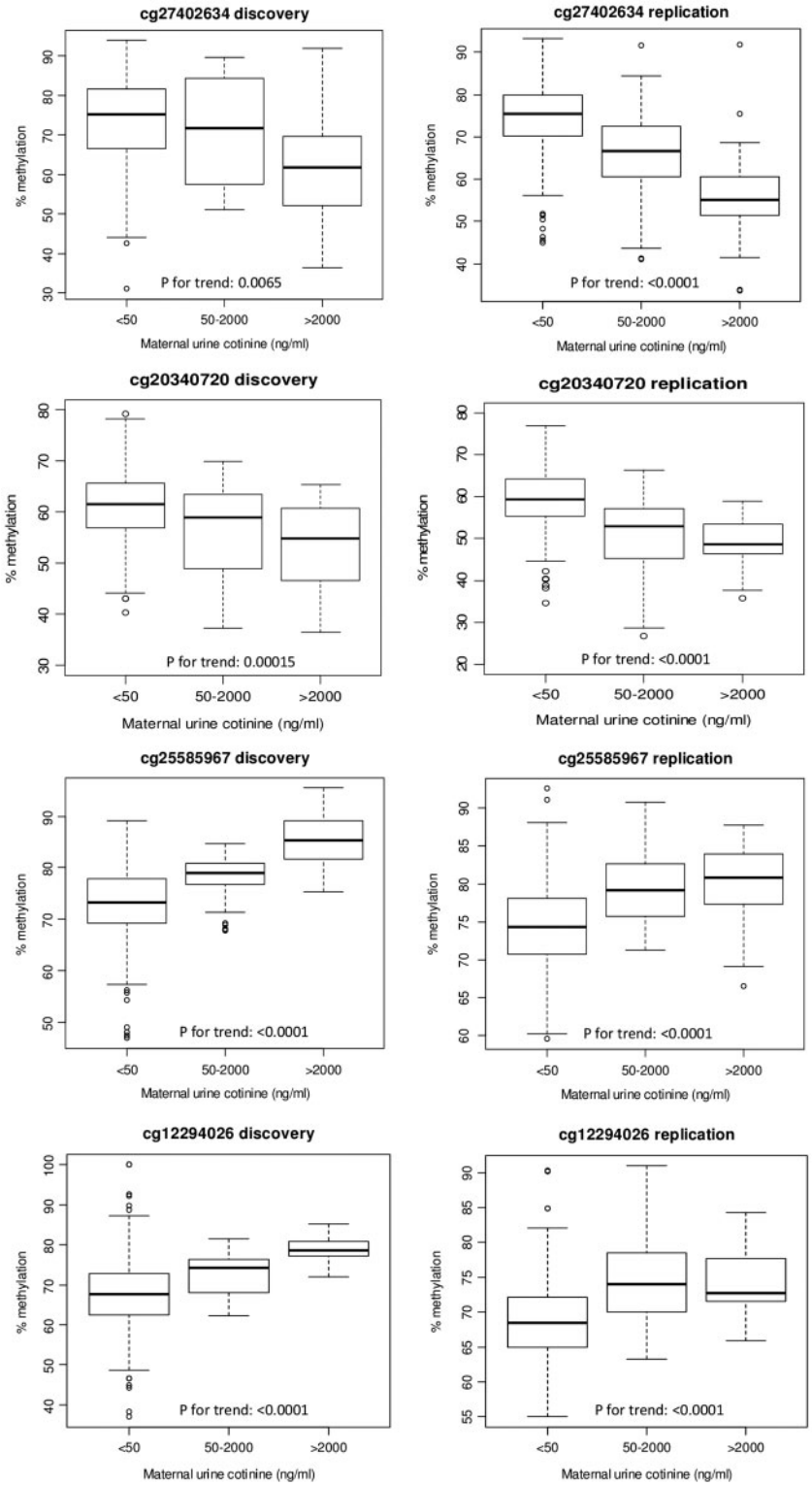

Figure 1. Relationship between maternal urine cotinine concentration (ng/ml) during pregnancy and placental methylation levels at top-ranking CpGs.

stress, phagocytosis, peroxisomal metabolism, and myometrial relaxation and contraction according to ConsensusPath (Supplementary Table 7, available as Supplementary data at *IJE* online). Although none of the Gene Ontology terms for biological processes showed an association at FDR threshold, some of them were related to placental development and stress (response to alcohol and hypoxia) (Supplementary Table 8, available as

Table 4. Mediation analysis examining the association between maternal smoking during pregnancy and birthweight of the offspring through differential placental methylation at CpGs (discovery sample)

	Bc	SEc	Pc	*R*-squared			
BW = maternal smoking + covariates	−314.0	78.1	8.78×10^{-05}	0.247			
BW = maternal smoking + CpG + covariates	Bc'	Bb	SEb	Pb	Diff. betas (Bc − Bc') g	Mediation % ((Bc – Bc')/Bc)	Sobel *P*-value
cg27402634	−199.5	1298.4	328.3	0.0001	−114.5	36.5	0.0002
cg20340720	−326.1	878.9	578.4	0.130	12.1	−3.9	0.138
cg25585967	−297.9	−1361.3	654.4	0.039	−16.1	5.1	0.048
cg12294026	−330.1	−1033.1	753.3	0.172	16.1	−5.1	0.189

Covariates: child's sex, area of study, gestational age, maternal age, maternal social class, parity and plate. The coefficients Bc and Bc' can be interpreted as the amount of grams lower birthweight for smoking vs non-smoking mothers in the 'smoking to birthweight' and full model, respectively. Bb represents the effect of methylation level (coded as a proportion between 0–1) on birthweight. For cg27402634, this means that an increase of 100% in methylation level is associated with 1298.4 g higher birthweight. Bc, effect estimate for smoking in the model: BW = Smoking + covariates; Bc', effect estimate for smoking in the model: BW = Smoking + CpG + covariates; Bb, effect estimate for CpG in the model: BW = CpG + covariates.

Table 5. Causal estimates for a 1% increase in methylation levels on birthweight *z*-score using genetic variants around the cg27402634 site region

	Causal estimate	Lower 95% CI	Upper 95% CI	*p*-value for heterogeneity
All five tags at a *p*-value < 5E-02 ($r^2 > 0.5$)	0.007	0.002	0.011	1.03E-01
All five tags at a *p*-value < 5E-02 ($r^2 > 0.5$), correction for LD	0.005	0.001	0.008	5.90E-02
All five tags at a *p*-value < 5E-02 ($r^2 > 0.5$), MR-Egger	0.006	−0.016	0.028	–
Two close tag SNPs surrounding cg27402634 at a *P*-value < 5E-02	0.007	0.002	0.012	1.59E-01

SNPs surrounding the cg27402634 site (250 kb up- and downstream) with a MAF > 1% and a quality of imputation > 0.8 were selected. Among them, only tag SNPs ($r^2 < 0.5$) associated with cg27402634 methylation levels at a *p*-value < 0.05 are shown. The association between genetic variants and methylation was obtained in this study using bisulfite pyrosequencing data ($n = 136$). The association between genetic variants and birthweight *Z*-scores was retrieved from GWAS summarized data from the Early Growth Genetics consortium (EGG) (http://egg-consortium.org/birth-weight.html). The Mendelian randomization approach was conducted using the LHR method, except for MR-Egger regression that used the inverse-variance weighted test. LD, linkage disequilibrium.

Supplementary data at *IJE* online). Our analysis showed less relevant pathways or biological functions when only CpGs annotated in genes were included (Supplementary Tables 9 and 10, available as Supplementary data at *IJE* online). Finally, we investigated the protein–protein interaction network using STRING (Supplementary Figure 4, available as Supplementary data at *IJE* online). PDE7B, ADCY7, PRKCE and PLCG2 proteins were found to be interconnected and three of them (ADCY7, PRKCE and PLCG2) mapped in the Myometrial Relaxation and Contraction Wikipathway.

Discussion

We investigated the effect of maternal smoking during pregnancy on DNA methylation signatures in placenta, a target organ controlling fetal growth and development. Our EWAS analysis revealed 50 CpGs, representing 46 loci, differentially methylated in smokers compared with non-smokers (FDR < 0.05). Fourteen of these loci showed a difference in methylation levels greater than 5%. Furthermore, we validated and replicated by bisulfite pyrosequencing the association between maternal smoking in pregnancy and placental methylation of three top-ranking loci (between *LINC00086* and *LEKR1*, *WBP1L* and *TRIO*) and confirmed dose–response relationships with maternal urine cotinine levels during pregnancy. Moreover, the use of paternal smoking as a negative control exposure supported an intrauterine effect of maternal smoking on DNA methylation changes in placenta. In addition, results from a two-sample MR study suggested a causal effect between decreases in placental methylation levels at cg27402634 and lower birthweight in the offspring. Finally, enrichment analysis pointed towards several functional pathways including signalling by growth factors, inflammation, oxidative stress, peroxisomal metabolism, and myometrial relaxation and contraction. To our knowledge, this is the largest study assessing the effect of maternal smoking during pregnancy on DNA methylation patterns in placenta on the epigenome-wide scale including a rigorous validation and replication of the main findings.

Among the 14 CpGs showing a differential methylation greater than 5% in relation to maternal smoking in pregnancy, we found some genes previously related to birthweight, asthma, type II diabetes, central nervous system disorders and some types of cancer (Supplementary Table 11, available as Supplementary data at *IJE* online). Interestingly, most of the identified CpGs have shown interactions with tobacco chemical compounds such as tetrachlorodibenzodioxin, several benzo(a)pyrenes and cotinine (Supplementary Table 11, available as Supplementary data at *IJE* online), strengthening our results. In accordance, enrichment analysis showed several functional pathways potentially related to adverse effects of toxic compounds (including tobacco smoke) such as inflammation, oxidative stress and peroxisomal metabolism.

Our most remarkable finding was lower placental methylation at cg27402634 in relation to both self-reported and cotinine-based evidence of maternal smoking exposure. Cg27402634 is located in a DNase hypersensitivity region that acts as a weak promoter between *LINC00086* and *LEKR1 (Leucine, Glutamate and Lysine Rich 1)* genes and showed no correlation with methylation levels at surroundings CpGs (Supplementary Figure 5, available as Supplementary data at *IJE* online). Moreover, differential methylation at cg27402634 mediated the association of maternal smoking during pregnancy and lower offspring birthweight. Interestingly, genetic variation downstream *LEKR1* has been previously linked to fetal growth, lower birthweight and placental weight[31,32] and increased child adiposity at birth.[33] Moreover, we used meQTLs as instrumental variables to assess the causal effect of methylation changes at cg27402634 on birthweight. We found that birthweight increased by 12.98 g per 1% increase in DNA methylation at the cg27402634 site. The causal estimate from the MR analysis was 0.007 SD units of birthweight *z*-score per 1% increase in methylation levels, which is equivalent to 3.36 g according to equivalences between *z*-score birthweight and grams.[32,34] This means that the estimate from the observational study (an increase of 12.98 g of birthweight per 1% increase in methylation) is four times higher than the causal estimate obtained from the MR approach.

We also validated the findings of two CpGs in *TRIO* (*Trio Rho Guanine Nucleotide Exchange Factor*). Methylation levels at these CpGs were correlated (Supplementary Figure 6, available as Supplementary data at *IJE* online) and mediated in part the association between maternal smoking and birthweight in the discovery sample. Furthermore, *TRIO* has been reported to interact with benzo(a)pyrenes, resulting in decreased gene expression,[35,36] which is in accordance with an increased methylation levels in relation to maternal smoking exposure found in the present study.

Differential methylation at cg20340720 located in intron 1 of *WBP1L* (*Domain Binding Protein 1-Like*) in relation to maternal smoking was also validated and replicated. Methylation levels at this CpG site were not correlated with nearby positions (Supplementary Figure 7, available as Supplementary data at *IJE* online). We did not find evidence that methylation at cg20340720 mediates the association between maternal smoking in pregnancy and offspring birthweight.

Furthermore, we identified several pathways involved in growth factor signalling and four of our top-ranking loci (*ADCY7*, *GUCA2B*, *PRKCE* and *PLCG2*) were mapped to a pathway related to myometrial transition to labour in mouse.[37] *ADCY7* and *GLUC2B* are involved in maintaining myometrial relaxation during pregnancy and are hypermethylated in placentas of smoker mothers, whereas *PRKCE* and *PLCG2* participate in the activation of labour work. Tobacco smoking during pregnancy is associated with shorter pregnancies[1] and smoking seems to increase oxytocin sensitivity of pregnant myometrium.[38] Whether alterations in these genes in response to tobacco smoking play a similar function in placenta, which is derived from the endometrium, needs further investigation.

In addition, we compared our results with previous findings from studies investigating DNA methylation changes at the epigenome-wide scale in relation to maternal smoking in pregnancy. We identified 10 studies reporting in total 440 unique CpGs differentially methylated (Supplementary Table 12, available as Supplementary data at *IJE* online) and 172 of them were identified in placental tissue. We did not detect overlap between smoking-sensitive CpGs reported in the literature in blood and placental tissue. Thirty-three out of 404 CpGs covered in our study were associated with maternal smoking at a *P*-value < 0.05 and 18 showed an association in the same direction as previously reported (Supplementary Table 12, available as Supplementary data at *IJE* online). The following genes were replicated in our study at *P*-value < 0.05 in the same direction in at least one assessed CpG: *AHRR*, *COX6CP4*, *GFI1*, *HBM* (reported only in placenta), *HLA-DPB2*, *MESP1*, *OR8B9P*, *PLCL2*, *PXN*, *SSH1*, *TPM3P2*, *TRIM59* and *ZFP62*. Three additional CpG sites previously reported in placenta (i.e. *VAMP8*, *CCDC64* and *DDR2*) were also identified in our study, but we could not check the direction of the association. Curiously, *CYP1A1* was hypomethylated in smokers in the present study in accordance with a previous study in placenta,[16] whereas it has been reported to be hypermethylated in blood of the offspring of smokers.[9–11]

We also evaluated the association of maternal tobacco smoking with placental methylation at six genes previously reported to account for 78% of the variance in birthweight.[39] *PGRMC1* was not recovered in the present study. Ten out of the 184 CpG sites evaluated showed differential methylation in relation to maternal tobacco smoking at a *P*-value < 0.05, but none of them survived after correction for multiple testing (Supplementary Table 13, available as Supplementary data at *IJE* online). All the investigated genes, except *RGS14*, showed differential methylation at a *P*-value < 0.05 in at least one CpG site in relation to maternal smoking.

Reasons for differences between studies include the limitations of comparing the 27K and 450K Illumina platforms, spurious findings due to small sample sizes and more likely the use of different target tissues. It is plausible that, to a large extent, altered methylation patterns in response to prenatal tobacco smoke exposure are quite tissue-specific, differing between placental tissue and blood collected at different lifetime points. In this sense, the top-ranking differentially methylated CpG in our study, located close to a *locus* previously reported to be associated with birthweight in GWAS studies, has not been previously reported in any of the EWAS performed in blood DNA, which highlights the importance of the target tissue in environmental epigenetic studies.

Strengths of our study include the use of placental tissue to assess DNA methylation as a potential mediator of adverse effects of maternal tobacco during pregnancy on offspring birthweight. Secondly, the application of the Infinium 450K BeadChip technology to assess genome-wide methylation profiles, which offers greatly improved genomic coverage over the earlier 27K platform. Third, findings of the three top-ranking loci were validated by bisulfite pyroquencing, the gold standard technique to quantify DNA methylation levels at single-nucleotide resolution[40] and, moreover, results were replicated in an independent population. Fourth, we confirmed dose–response relationships between methylation levels of CpGs and maternal urine cotinine levels in pregnancy—a well-validated biomarker for tobacco smoke. Finally, we took into account in the analyses the potential effect of paternal tobacco smoking on placental methylation and demonstrated consistently stronger maternal associations, providing further evidence for intrauterine effects in three out of the four top-ranked hits. Although attenuated, paternal smoking remained associated with methylation levels at cg20340720 after adjustment for maternal smoking. This could be explained by residual confounding by shared familiar environmental factors and/or by genetic factors. Nevertheless, mutually adjusted models supported an intrauterine effect of maternal smoking on lower DNA methylation levels at cg27402634.

The study has also some limitations. Our discovery study was based on a reduced sample size of smokers ($n = 28$), which may limit the power to detect methylation differences in additional regions of the genome and increase chances for false-positive and false-negative findings. We cannot rule out potential bias due to cell-type mixture in placental tissue or maternal contamination. Moreover, only one biopsy per sample was done for DNA extraction, which could have not accounted for potential regional variation in placental DNA methylation; however, some evidence suggests that this would account for a minor source of variation in placenta tissue.[41] In addition, findings of the mediation analysis could not be replicated, as maternal smoking was not associated with offspring birthweight in the replication sample. Finally, the results of the MR approach should be taken with caution because the instrumental variables (meQTLs) were generated within our own dataset, which could result in bias of the causal estimate. Unfortunately, we are not aware of previous studies describing meQTLs in placenta. Moreover, pleiotropic effects of meQTLS on birthweight cannot be completely ruled out. If present, meQTLs could have a direct effect on birthweight resulting in changes in DNA methylation levels (reverse causation).

In conclusion, we identified novel loci differentially methylated in placenta in relation to maternal smoking during pregnancy. Findings of the three top-ranking loci were validated and replicated by bisulfite pyrosequencing. We found suggestive evidence that intrauterine exposure to maternal tobacco smoking decreases methylation levels at cg27402634 (between *LINC00086* and *LEKR1*), which leads to lower birthweight of the offspring. Our results indicate that maternal smoking during pregnancy impacts specific placental methylation profiles and highlight the importance of the target tissue in epigenetic studies. The investigation of the mechanistic roles that the identified differentially methylated loci may play in mediating the association between maternal smoking during pregnancy and offspring phenotypes later in life is warranted.

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