

## Original Contribution

# Mediation by Placental DNA Methylation of the Association of Prenatal Maternal Smoking and Birth Weight

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Prenatal maternal smoking is a risk factor for lower birth weight. We performed epigenome-wide association analyses of placental DNA methylation (DNAm) at 720,077 cytosine-phosphate-guanine (CpG) sites and prenatal maternal smoking among 441 mother-infant pairs (2010–2014) and evaluated whether DNAm mediates the association between smoking and birth weight using mediation analysis. Mean birth weight was 3,443 (standard deviation, 423) g, and 38 mothers (8.6%) reported smoking at a mean of 9.4 weeks of gestation. Prenatal maternal smoking was associated with a 175-g lower birth weight (95% confidence interval (CI): –305.5, –44.8) and with differential DNAm of 71 CpGs in placenta, robust to latent-factor adjustment reflecting cell types (Bonferroni-adjusted  $P < 6.94 \times 10^{-8}$ ). Of the 71 CpG sites, 7 mediated the association between prenatal smoking and birth weight (on *MDS2*, *PBX1*, *CYP1A2*, *VPRBP*, *WBP1L*, *CD28*, and *CDK6* genes), and prenatal smoking  $\times$  DNAm interactions on birth weight were observed for 5 CpG sites. The strongest mediator, cg22638236, was annotated to the *PBX1* gene body involved in skeletal patterning and programming, with a mediated effect of 301-g lower birth weight (95% CI: –543, –86) among smokers but no mediated effect for nonsmokers ( $\beta = -38$  g; 95% CI: –88, 9). Prenatal maternal smoking might interact with placental DNAm at specific loci, mediating the association with lower infant birth weight.

DNA methylation; epigenetics; mediation; smoking

Abbreviations: CDK6, cyclin dependent kinase 6; CI, confidence interval; CpG, cytosine-phosphate-guanine; CYP1A2, cytochrome P450 family 1 subfamily A member 2; DNAm, DNA methylation; EWAS, epigenome-wide association studies; FDR, false discovery rate; Gen3G, Genetics of Glucose Regulation in Gestation and Growth; PBX1, pre-B-cell leukemia homeobox 1 protein; SD, standard deviation; VPRBP, Vpr (HIV-1) binding protein; WBP1L, WW domain binding protein 1-like.

**Editor's note:** An invited commentary on this article appears on page 1887, and the authors' response appears on page 1890.

Prenatal maternal smoking is an established risk factor for lower birth weight and reduced fetal growth, and it is associated with increased risk of major childhood diseases such as asthma, obesity, and cancers (1–3). A complex chemical mixture of over 7,000 compounds make up cigarette smoke, including known reproductive toxicants such as carbon monoxide, arsenic, lead, cadmium, and nicotine. Although prenatal maternal cigarette smoke is a well-recognized risk factor for reduced fetal growth, mechanisms are only partially understood and might include

both direct effects on the fetus and morphological and functional changes to the placenta (4).

DNA methylation (DNAm), occurring mostly at cytosine-phosphate-guanine dinucleotides (CpG sites), can involve extensive erasure, rewriting, and reprogramming during embryogenesis and cellular differentiation (5). This reprogramming phase might pose a susceptibility window for early nutritional and environmental cues to program future health trajectories of newborns by altering cellular structure, lineage commitment, physiology, and metabolic functions, a hypothesis known as fetal programming (6). For instance, prenatal smoking has been consistently associated with altered cord blood DNAm of several genes (7–10). Previous studies have shown that cord blood DNAm variability associated with prenatal

smoking partially mediates the association of smoking and lower birth weight (11, 12). While multiple studies have looked at maternal smoking and DNAm in cord blood, few have investigated placental DNAm variation, and even fewer have used an agnostic epigenome-wide approach (13–16).

Methylation of DNA is vastly cell-type specific, and early environmental influences on the epigenome are likely tissue- and cell-type specific (17). Thus, investigating the role of target tissue in epigenetic studies is critical (18). The placenta is the master regulator of the fetal environment, with active endocrine and metabolic functions, synthesizing hormones and regulating nutrient and waste exchange (19). Nicotine, a parasymphomimetic stimulant and primary constituent of tobacco smoke, acts as a vasoconstrictor reducing intervillous placental blood flow, increases hypoxia, and interferes with placental development (20, 21). Additionally, many other reproductive toxicants present in cigarette smoke cross the placenta, exposing the fetus, and some accumulate within placental structures (22). Consequently, the placenta might be particularly susceptible to prenatal tobacco smoke and a target tissue to mediate associations with fetal growth. In this study, we hypothesized that DNAm variability of the placenta associated with prenatal maternal smoking early in pregnancy would mediate the established association of prenatal smoking and lower infant birth weight.

## METHODS

### Study population

Study participants were selected from the Genetics of Glucose Regulation in Gestation and Growth (Gen3G) cohort, a prospective prebirth cohort recruited between January 2010 and February 2014 in Sherbrooke, Canada. We recruited expecting mothers during the first trimester of pregnancy. They were eligible for enrollment if they were 18 years of age or older, had a singleton pregnancy, did not have prepregnancy diabetes based on medical history and screening, and planned to deliver at the Centre Hospitalier Universitaire de Sherbrooke. For the present study, mother-infant pairs were selected if they consented to placental tissue collection for DNA isolation and had >37 weeks of gestation at delivery.

The Gen3G cohort has been previously described (23). Mothers provided written informed consent prior to enrollment in accordance with the Declaration of Helsinki. Study protocols were approved by the ethical review board from the Center Hospitalier Universitaire de Sherbrooke.

### Placental tissue and DNA

Placental tissue from the fetal side was collected by trained staff within 30 minutes of delivery. A 1-cm<sup>3</sup> sample of placental tissue was collected approximately 5 cm from the umbilical cord insertion. Placental samples were subsequently stored at –80°C until DNA extraction occurred. We purified DNA from placental samples using the All Prep DNA/RNA/Protein Mini Kit (Qiagen, Germantown, Maryland). Purity of extracted DNA was evaluated using a spectrophotometer (Ultrospec 2000 UV/Visible; Pharmacia Biotech, Piscataway, New Jersey).

### Prenatal maternal smoking and birth weight

During the first research visit, occurring at a mean of 9.4 (standard deviation (SD), 2.3) weeks of gestation, trained staff administered standardized questionnaires to participating women to collect medical and lifestyle history, including smoking behavior. Participants were classified as current smokers or not current smokers (which included past smokers) based on self-report at this visit. Within 2 hours of delivery, birth weight was measured in grams with an electronic scale in the hospital, following standard clinical protocol, by obstetrical nurses.

### Placental DNA methylation

Epigenome-wide DNAm measurements were performed on placental DNA and quantified using the Infinium MethylationEPIC BeadChip (Illumina, San Diego, California), measuring over 850,000 CpGs at a nucleotide resolution. Data processing and cleaning have been described previously and in the Web Appendix (available at <https://academic.oup.com/aje>) (24).

### Statistical analyses

We report demographic characteristics using means and standard deviations or proportions. We conducted epigenome-wide association studies (EWAS) of self-reported prenatal maternal smoking behavior by fitting linear regression models using *limma* for each CpG on the  $\beta$ -value scale. Confounding factors were selected a priori based on EWAS recommendations (25). We controlled for exposure-mediator (maternal age), mediator-outcome (sex, parity, gestational age, and cell-type/DNAm heterogeneity), and exposure-outcome (maternal body mass index, calculated as weight (kg)/height (m<sup>2</sup>)) confounding (Web Figure 1). We examined models adjusting for cell-type/DNAm heterogeneity estimated using latent-factors from *ReFACTor* (26), a reference-free adjustment method, using the first 10 principal components selected by visual inspection (Web Figure 2). We used quantile-quantile plots of *P* values from EWAS to inspect genomic inflation and calculated genomic inflation factors ( $\lambda$ ). We used Manhattan and volcano plots to report EWAS results. We selected CpGs as candidate mediators if they were associated with prenatal maternal smoking or birth weight in both crude and *ReFACTor*-adjusted models (Bonferroni-adjusted  $P < 6.94 \times 10^{-8}$ ). We selected candidate CpG mediators found in both crude and *ReFACTor*-adjusted models to ensure reproducibility, given that reference-free methods have varying assumptions (27).

For each individual candidate CpG mediator, we fitted 2 linear regression models: 1) the mediator model, with DNAm of the CpG as the outcome and prenatal smoking as a predictor, adjusting for maternal age, body mass index, parity, gestational age, sex, and the 10 principal components from *ReFACTor*; and 2) the outcome model, with birth weight as the outcome and prenatal smoking as a predictor, adjusting for the mediator, DNAm of the CpG, and the covariates from the first model. In mediation analyses, we adjusted for multiple comparisons using a false discovery rate (FDR) of 5% (<0.05) for the average causal mediated effect tests. Among the CpGs selected (FDR < 0.05), we tested for the exposure-mediator interaction using  $P < 0.10$ . To test for statistical mediation, we used a

counterfactual framework to estimate the direct and indirect effects. This method is implemented in the *mediation* package in R (R Foundation for Statistical Computing, Vienna, Austria) (28). We used 100,000 simulations to report estimates and 95% confidence intervals.

Additionally, we tested for mediation among 3 CpGs previously reported to mediate the association of smoking and birth weight in placenta (16). For mediators found in our placenta analyses, we tested for mediation on 437 paired cord blood samples from our same cohort also measured with the MethylationEPIC array.

Our approach to mediation analysis assumes the sequential ignorability assumption: that there is no unmeasured confounding of the exposure-outcome, mediator-outcome, exposure-mediator relationships and that none of the mediator-outcome confounders are affected by the exposure (29). We visually inspected violation of this assumption by estimating correlations ( $\rho$ ) between the residuals of the mediator and outcome regression for which direct and indirect effects would be zero. We checked for outliers and assumptions of linear regression models for mediation using regression diagnostic plots; all models reasonably met assumptions.

Among all candidate CpG mediators, we conducted *KEGG* biological pathway enrichment analyses using *missMethyl* with probe density bias correction (30). We also performed enrichment of candidate CpG mediators to test the likely tissue/cell of origin of findings using eFORGE (<https://eforge.altiusinstitute.org/>). We performed data analyses using R, version 3.5.1 (R Foundation for Statistical Computing).

## RESULTS

We analyzed a total of 441 mother-infant pairs. At enrollment, mean maternal age was 28.3 (SD, 4.3) years and body mass index was 25.4 (SD, 5.6). All pregnant women were white, 48.5% were primiparous, and 38 (8.6%) reported currently smoking at the first research visit. Of the newborns, 52.6% were male; the mean gestational age was 39.5 (SD, 1.04) weeks, and mean birth weight was 3,443 (SD, 422.7) g (Table 1). Prenatal maternal smoking was associated with a 175-g lower birth weight (95% confidence interval (CI): -305.5, -44.8) after adjustment for maternal age, body mass index, parity, gestational age, sex, and cell-type/DNAM heterogeneity. Results remained consistent in crude and partially adjusting models or when using birth-weight-for-gestational-age z scores (Web Table 1).

Prenatal maternal smoking was associated with placental DNAm at 89 CpGs (Bonferroni-adjusted  $P < 6.94 \times 10^{-8}$ ) in models adjusting for maternal age, body mass index, parity, gestational age, and sex. Further adjustment for cell-type/DNAM heterogeneity yielded 153 differentially methylated CpGs (Bonferroni-adjusted  $P < 6.94 \times 10^{-8}$ ) (Figure 1A; Web Figure 3). Effect sizes ranged from 29.8% lower DNAm (cg27402634; the leucine, glutamate, and lysine rich 1 gene (*LEKRI*)) to 16.5% greater DNAm (cg11051192; polyhomeotic homolog 1 (*PHCI*)) in the placenta of prenatal smokers compared to nonsmokers (Figure 1B).

For mediation analyses, we tested 71 CpGs individually as candidate mediators found to be differentially methylated (Bonferroni-adjusted  $P < 6.94 \times 10^{-8}$ ) in crude models and

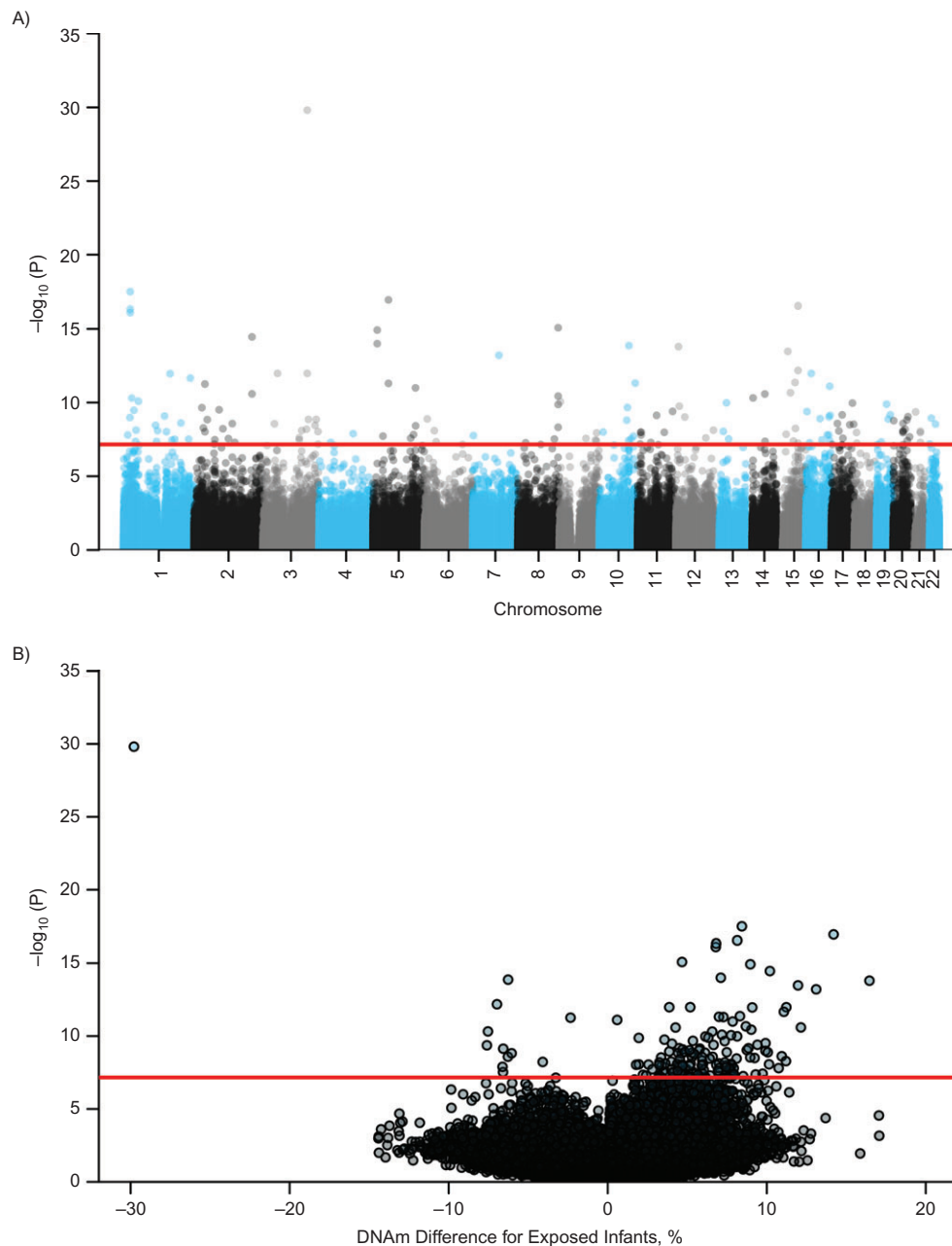
**Table 1.** Participant Characteristics From the Genetics of Glucose Regulation in Gestation and Growth Study ( $n = 441$ ), Sherbrooke, Canada, 2010–2014

Characteristic	No.	%	Mean (SD)
Maternal age, years			28.3 (4.3)
Maternal body mass index <sup>a</sup>			25.4 (5.6)
Primiparous	214	48.5	
White ethnicity	441	100	
Smoking during pregnancy			
No	403	91.4	
Yes	38	8.6	
Child sex			
Male	232	52.6	
Female	209	47.4	
Gestational age, weeks			39.5 (1.04)
Birth weight, g			3,443 (422.7)
Birth weight for gestational age, z score			0.39 (0.93)

<sup>a</sup> Weight (kg)/height (m)<sup>2</sup>.

models adjusting for cell-type/DNAM heterogeneity. The 71 CpGs were annotated to genes enriched (FDR  $< 0.05$ ) within the notch signaling pathway, pathways in cancer, and the human papillomavirus infection pathway (Web Table 2). The 71 CpGs associated with smoking were enriched in DNase I hypersensitivity sites of fetal placental tissues (Web Figure 4). Genomic annotation and summary results from fully adjusted EWAS analyses of prenatal smoking for the 71 CpGs considered as candidate mediators are reported in Web Table 3.

We found 7 CpGs mediating the association between prenatal maternal smoking and birth weight (FDR  $< 0.05$ ; adjusted for 71 comparisons). Among these 7 CpGs, we observed exposure-mediator interaction for 5 ( $P < 0.10$ ), as shown in Table 2. As shown in Table 3, among mediators interacting with prenatal smoking, cg14160212 (located within 151 base pairs of the myelodysplastic syndrome 2 translocation-associated protein (*MDS2*) gene) had a mediated effect of 280-g lower birth weight (95% CI: -478, -102) for smokers but no mediating effect for nonsmokers. Similarly, cg22638236 annotated to the pre-B-cell leukemia homeobox 1 protein gene (*PBX1*) had a mediated effect of 301-g lower birth weight (95% CI: -543, -86) for smokers but no mediated effect for nonsmokers. DNAm of cg20385913 (within 5,569 base pairs of the cytochrome P450 family 1 subfamily a member 2 (*CYP1A2*) gene) had a mediated effect of 244-g lower birth weight (95% CI: -457, -49) for smokers and no mediating effect for nonsmokers. DNAm of cg11280108 (located within 3,609 base pairs of Vpr (HIV-1) binding protein (*VPRBP*)) had a mediated effect of 185-g lower birth weight (95% CI: -363, -25) for smokers and no mediated effect in nonsmokers. Last, cg01883283 annotated to the WW domain binding protein 1-like gene (*WBP1L*) had a mediated effect of 135-g lower birth weight (95% CI: -246, -34) among smokers only. As noted in Table 2, no exposure-mediator interaction was observed for cg23878024, with an average mediated effect of 86-g lower birth weight (95% CI: -144, -34), and



**Figure 1.** Manhattan plot (A) and volcano plot (B) for epigenome-wide association analyses of prenatal maternal smoking and placental DNA methylation (DNAm), adjusted for confounders and cell-type/DNA methylation heterogeneity, Sherbrooke, Canada, 2010–2014. The red line is the Bonferroni threshold for statistical significance.

cg24005876 annotated to the cyclin dependent kinase 6 (*CDK6*) gene body, with an average mediated effect of 91-g lower birth weight (95% CI:  $-149, -41$ ).

Distribution of DNAm levels among the 7 mediating CpGs are shown in raincloud plots (Web Figure 5) and according to smoking status on boxplots (Web Figure 6). Mediation analysis using birth-weight-for-gestational-age  $z$  scores as the outcome, instead of birth weight, yielded consistent results (Web Table 4).

Using 437 paired cord blood samples, we did not observe mediated effects at the 7 CpGs (Web Table 5).

For comparability, we examined CpGs previously reported to mediate smoking–birth weight associations by placental DNAm in, to our knowledge, the only EWAS performed to date (16) and observed a consistent average causal mediated effect of 145-g lower birth weight (95% CI:  $-258, -36$ ) for cg27402634 annotated to the leucine, glutamate, and lysine rich

**Table 2.** Placental DNA Methylation Mediators for the Association of Prenatal Maternal Smoking and Infant Birth Weight With a Significant Average Mediated Effect, Sherbrooke, Canada, 2010–2014

CpG Site	Annotation				Average Direct Effects			Average Mediated Effects			Exposure Mediator Interaction
	Chromosome	Position	Gene	Distance in Base Pairs <sup>a</sup>	$\beta$	95% CI	P	$\beta$	95% CI	FDR <sup>b</sup>	P Value
cg14160212	1	23907834	<i>MDS2</i> <sup>a</sup>	151	-14 g	-179, 156	0.87	-153 g	-260, -57	0.05	0.005
cg22638236	1	164759974	<i>PBX1</i>	Body	-11 g	-180, 165	0.89	-169 g	-297, -58	0.05	0.02
cg20385913	15	75054510	<i>CYP1A2</i> <sup>a</sup>	5,569	-44 g	-207, 122	0.60	-139 g	-253, -36	0.03	0.04
cg11280108	3	51537627	<i>VPRBP</i> <sup>a</sup>	3,609	-69 g	-221, 85	0.37	-115 g	-211, -30	0.03	0.09
cg01883283	10	104512669	<i>WBP1L</i>	Body	-86 g	-230, 57	0.24	-86 g	-152, -27	0.04	0.09
cg23878024	2	204488580	<i>CD28</i> <sup>a</sup>	82,618	-89 g	-228, 49	0.21	-86 g	-144, -34	0.03	0.12
cg24005876	7	92303485	<i>CDK6</i>	Body	-84 g	-221, 53	0.23	-91 g	-149, -41	0.04	0.30

Abbreviations: CD28, CD28 molecule; CDK6, cyclin dependent kinase 6; CI, confidence interval; CpG, cytosine-phosphate-guanine; CYP1A2, cytochrome P450 family 1 subfamily A member 2; FDR, false discovery rate; MDS2, myelodysplastic syndrome 2 translocation-associated protein; PBX1, pre-B-cell leukemia homeobox 1; VPRBP, Vpr (HIV-1) binding protein; WBP1L, WW domain binding protein 1-like.

<sup>a</sup> Not within the gene; therefore, annotated distance to nearest gene.

<sup>b</sup> FDR-adjusted P value.

1 gene (*LEKRI*). Additionally, we observed DNAm  $\times$  smoking interaction on birth weight for this site. The mediated effect among smokers was 276-g lower birth weight (95% CI: -483, -76), with no mediated effect for nonsmoking mothers. We did not find a significant mediated effect for the other 3 CpGs reported by Morales et al. (16), possibly due to single-nucleotide polymorphisms within the probes (cg20340720, cg12294026) that might differ by minor allele frequency (Web Tables 6 and 7). Another important difference is that we used the MethylationEPIC array covering over 400,000 new CpGs located mostly at regulatory elements compared to the 450K previously used (31).

In sensitivity analyses of the mediated effect estimates, the sequential ignorability assumption might be violated for residual correlations of the mediator and outcome regressions far from the observed estimated mediated effects for all 7 CpGs (Web Figure 7).

## DISCUSSION

Prenatal maternal smoking is an established risk factor for lower birth weight. Here we report our finding that placental DNAm of genes associated with prenatal maternal smoking mediated the association with reduced birth weight. We observed interaction with 5 of the 7 CpGs found. This resulted in a strong mediated effect among smokers and null, attenuated effects for nonsmokers for 5 out of the 7 CpGs. Our results suggest that DNAm variability in the placenta associated with prenatal maternal smoking might play an active role in the well-established association of prenatal smoking and low birth weight. Our results also highlight the need to consider exposure-mediator interactions in epigenetic studies as a relevant biological and statistical model.

We observed several exposure-mediator interactions suggesting that prenatal maternal smoking must be present for the

**Table 3.** Estimates for the Direct and Causal Mediated Effects Among Cytosine-Phosphate-Guanine Sites With Evidence of Exposure-Mediator Interaction According to Prenatal Maternal Smoking Status, Sherbrooke, Canada (2010–2014)

CpG Site	Gene	Prenatal Maternal Smoking						No Prenatal Maternal Smoking					
		Direct Effects			Mediated Effects			Direct Effects			Mediated Effects		
		$\beta$	95% CI	P Value	$\beta$	95% CI	P Value	$\beta$	95% CI	P Value	$\beta$	95% CI	P Value
cg14160212	<i>MDS2</i>	-141 g	-296, 11	0.07	-280 g	-478, -102	0.001	114 g	-110, 339	0.31	-25 g	-83, 30	0.38
cg22638236	<i>PBX1</i>	-142 g	-302, 14	0.07	-301 g	-543, -86	0.005	121 g	-125, 369	0.33	-38 g	-88, 9	0.11
cg20385913	<i>CYP1A2</i>	-148 g	-302, 2	0.05	-244 g	-457, -49	0.01	61 g	-169, 291	0.60	-35 g	-94, 22	0.23
cg11280108	<i>VPRBP</i>	-139 g	-285, 6	0.06	-185 g	-363, -25	0.02	1 g	-198, 201	0.99	-45 g	-96, 2	0.06
cg01883283	<i>WBP1L</i>	-135 g	-278, 8	0.06	-135 g	-246, -34	0.008	-38 g	-203, 127	0.65	-38 g	-97, 18	0.19

Abbreviations: CI, confidence interval; CpG, cytosine-phosphate-guanine; CYP1A2, cytochrome P450 family 1 subfamily A member 2; MDS2, myelodysplastic syndrome 2 translocation-associated protein; PBX1, pre-B-cell leukemia homeobox 1 protein; VPRBP, Vpr (HIV-1) binding protein; WBP1L, WW domain binding protein 1-like.

mediator, placental DNAm, to affect birth weight. This is a prime example of a mediated interaction, plausible for biological mechanisms and models (32). A mediated-interaction epigenetic model is supported by previous studies that demonstrate epigenetic malleability associated with environmental exposures. For example, we have found that epigenetic variability associated with prenatal environmental exposures at birth might be attenuated as children age (33, 34). Additionally, in a randomized trial it was demonstrated that DNAm variability associated with prenatal maternal smoking in placenta, cord blood and buccal cells can be restored to “normal” DNAm levels comparable to that of infants born to nonsmoking mothers after vitamin-C supplementation (35). This reversibility of the mediated effect supports the hypothesis that prenatal maternal smoking must be present for DNAm to affect birth weight. This concept of mediated interaction has been proposed to more accurately reflect phenomena observed in models of disease (32). We propose that a mediated-interaction model is highly relevant to epigenetic studies.

The strongest CpG mediator was annotated to *PBX1*. *PBX1* encodes a nuclear protein that belongs to the PBX homeobox family of transcriptional factors, widely transcribed throughout the developing murine embryo and essential for skeletal patterning and programming (36). The *PBX1* gene regulates osteoblast differentiation by altering chromatin structures and regulating transcription of bone specific genes (37). A previous EWAS of cord blood reported greater DNAm of a genomic region in *PBX1* to be strongly associated with higher birth weight for gestational age (38). In adults, higher urinary nicotine equivalents among smokers were associated with greater leukocyte DNAm at CpGs near *PBX1* (39). In a large study of adults, *PBX1* DNAm of leukocytes associated with current body mass index across multiple cohorts, and this association was replicated for DNAm of adipose tissue (40). Whether or not *PBX1* DNAm of placenta at birth is relevant for future child adiposity remains to be tested.

Prenatal smoking was also associated with 6.3% lower DNAm at cg01883283 annotated to *WBPIL* and mediated associations with birth weight. Placental DNAm of this gene has been linked to prenatal smoking at a nearby CpG within 145 base pairs of our site (8.7% lower DNAm at cg20340720) (16). However, in this previous study, no evidence of a mediated effect between prenatal smoking and birth weight was found for cg20340720. Another CpG mediator, cg24005876, annotated to the cell division protein kinase 6 (*CDK6*) gene, is member of the serine/threonine protein kinases family and a critical regulator of cell cycle. The *CDK6* gene has been shown to play an important role in the development of polyploidy as well as stromal decidualization (41). In a study of human term placentas, *CDK6* mRNA was modestly correlated with infant birth weight ( $r = 0.33$ ) (42). This gene also covers a susceptibility loci for lung cancer shown to interact with smoking status/history (43).

*CYP1A2* is involved in the metabolism of pharmacological compounds as well as polycyclic aromatic hydrocarbons (PAHs) present in cigarette smoke (44). The *CYP1A2* enzyme is induced by tobacco smoke, shown to be primarily mediated by aryl hydrocarbon receptor ligands like polycyclic aromatic hydrocarbons (45). Placental *CYP1A2* expression has been detected in first trimester but not full-term placentas (46). From candidate gene studies, lower placental DNAm of the *CYP1A1*

gene, another CYP family member, has been consistently associated with prenatal smoking (47–49). Additionally, prenatal maternal smoking has been consistently reported to be associated with differential DNAm near *CYP1A1* in cord blood analyses, as well as in an EWAS of placenta (9, 16).

Less is known about DNAm of cg11280108 within 3,611 base pairs of the *Homo sapiens VPRBP* gene but this region has been shown to be involved in cell proliferation, chromatin remodeling, and oocyte survival and programming (50–52). Much less is known about DNAm or placental expression of cg14160212 (myelodysplastic syndrome 2 translocation-associated protein (*MDS2*)) or cg23878024 (*CD28*), and these CpGs did not directly annotate to genes. The underlying genetic architecture could also influence observed results. However, only one of our mediators (cg01883283; *WBPIL*) was located within 500 kb of a birth-weight single-nucleotide polymorphism (rs74233809) (53).

A previous study performed an EWAS of prenatal maternal smoking and placental DNA, investigating the mediated effect of DNAm (16). In this study, cg27402634 (*LEKRI*) explained up to 36% of the association between prenatal smoking and lower birth weight. In our cohort, cg27402634 had the strongest association with prenatal smoking but did not reach an FDR < 0.05 for the average mediated effect. We did observe a similar mediated effect of 145-g lower birth weight post hoc, but contrary to the previous study, an exposure-mediator interaction was present at this site. Although we did not observe mediation for the other 2 CpGs reported by Morales et al. (16) (cg25585967 and cg12294026) within the trio rho guanine nucleotide exchange factor (*TRIO*) gene body, they were consistently associated with greater placental DNAm for smokers with similar magnitudes.

Our results highlight that prenatal maternal smoking signatures of placenta DNAm are distinct of cord blood signatures. For example, using paired cord blood samples from our study we found no evidence of mediation at the 7 discovered CpGs from placenta. We compared the 71 CpGs associated with maternal smoking in placenta to 6,073 CpGs (8) previously reported from meta-analyses of cord blood and found that only 1 CpG (cg21992501; tetratricopeptide repeat domain 27 (*TTC27*)) positively associated with smoking in our placenta samples and in the meta-analyses of cord blood.

Our study has several important limitations. In DNAm studies with exposure misclassification, it has been demonstrated that DNAm of CpGs could serve as better biomarkers of the exposure than self-report, thereby increasing the type I error rate, underestimating the direct effect, and overestimating mediated effects (54). We also did not collect objective exposure biomarkers, and it is possible that our estimated mediated effects are inflated. However, self-reported smoking during pregnancy had high sensitivity (97.6%) and specificity (100%) compared with plasma cotinine in a comparable Canadian population (55) and other large cohorts (56). Another important limitation is that we did not ascertain sustained smoking throughout pregnancy—women could have stopped smoking after the first research visit—or exposure to secondhand smoke. Future studies should consider the impact of periconceptional paternal smoking and its impact on germ cell DNAm and on the infant’s epigenome. Timing of exposure, mediator, and outcome ascertainment is an important limitation of our study—women could have continued to

smoke throughout pregnancy and placental DNAm levels could have changed until birth, when birth weight was measured. However, in the general Canadian population, approximately 53% of women quit smoking by the third trimester of pregnancy (57), so it is likely that most women in our study quit by the third trimester. Additionally, placental DNAm profiles are largely established in early gestation. Our assumptions are that smoking in early pregnancy influences DNAm of placental genes that are established in early gestation, thereby influencing fetal growth. Nevertheless, it is possible that fetal growth influences placental DNAm. Therefore, our results must be interpreted in light of the timing of exposure and DNAm measurements.

An important strength of our approach is the implementation of the counterfactual framework in mediation analysis to estimate effects in the presence of exposure-mediator interactions as a relevant biological model for epigenetic epidemiology. Nevertheless, statistical mediation might not accurately reflect underlying causal biological processes such as transcriptional memory or cell lineage commitment. Therefore, our results do not necessarily reflect a causal biological mechanism. Future studies could also develop epigenetic biosensors of prenatal smoking from placenta DNAm signatures that might serve as biomarkers of the prenatal environment.

In conclusion, in this cohort, we observed that placental DNAm variability at specific loci associated with prenatal maternal smoking might mediate the association with lower birth weight within biologically relevant genes. Placental DNAm mediators annotated to the *PBX1* gene, involved in skeletal patterning and programming; *CYP1A2* gene, whose activity is induced by polycyclic aromatic hydrocarbons found in cigarette smoke; *CDK6*, whose expression in placenta has been associated with birth weight and stromal decidualization; and the *WPIL* gene, previously shown to be differentially methylated in the placenta of smokers. Finally, we report evidence of significant exposure  $\times$  mediator interactions and propose this to be highly relevant model for epigenetic epidemiology.

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