

Fetal lung and placental methylation is associated with in utero nicotine exposure

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Abbreviations: CHR, chromosome; GTF2H2C, General transcription factor IIH, polypeptide 2C; GTF2H2D, General transcription factor IIH, polypeptide 2D; IUS, in utero smoke; JAK2, Janus Kinase 2; KEGG, Kyoto Encyclopedia of Genes and Genomes.

In utero smoke exposure has been shown to have detrimental effects on lung function and to be associated with persistent wheezing and asthma in children. One potential mechanism of IUS effects could be alterations in DNA methylation, which may have life-long implications. The goal of this study was to examine the association between DNA methylation and nicotine exposure in fetal lung and placental tissue in early development; nicotine exposure in this analysis represents a likely surrogate for in-utero smoke. We performed an epigenome-wide analysis of DNA methylation in fetal lung tissue ($n = 85$, 41 smoke exposed (48%), 44 controls) and the corresponding placental tissue samples ($n = 80$, 39 smoke exposed (49%), 41 controls) using the Illumina HumanMethylation450 BeadChip array. Differential methylation analyses were conducted to evaluate the variation associated with nicotine exposure. The most significant CpG sites in the fetal lung analysis mapped to the *PKP3* ($P = 2.94 \times 10^{-03}$), *ANKRD33B* ($P = 3.12 \times 10^{-03}$), *CNTD2* ($P = 4.9 \times 10^{-03}$) and *DPP10* ($P = 5.43 \times 10^{-03}$) genes. In the placental methylome, the most significant CpG sites mapped to the *GTF2H2C* and *GTF2H2D* genes ($P = 2.87 \times 10^{-06} - 3.48 \times 10^{-05}$). One hundred and one unique CpG sites with P -values < 0.05 were concordant between lung and placental tissue analyses. Gene Set Enrichment Analysis demonstrated enrichment of specific disorders, such as asthma and immune disorders. Our findings demonstrate an association between in utero nicotine exposure and variable DNA methylation in fetal lung and placental tissues, suggesting a role for DNA methylation variation in the fetal origins of chronic diseases.

Introduction

Public health efforts have not succeeded in eliminating cigarette smoking in the developed world, as reflected by the fact that 19% of the adult population in the United States continues to smoke cigarettes, with the prevalence being 16.5% in women 18 years and older.¹ The 2008 Pregnancy Risk Assessment and Monitoring System (PRAMS) data from 29 states suggests that approximately 13% of women (i.e., 1 in every 8) reported smoking during the last 3 months of pregnancy.² In utero smoke (IUS) exposure has been linked to multiple detrimental effects on the developing fetus,³⁻⁶ and a number of epidemiological studies have shown its aberrant influence on lung function.⁷⁻⁹ In addition to its impact on lung function, there is evidence of increased wheezing¹⁰⁻¹² and asthma associated with prenatal smoke exposure.^{11,13-16} In asthmatic subjects, a history of IUS exposure has been associated with reduced efficacy of inhaled

corticosteroids on decreasing airway responsiveness.¹⁷ In addition to influencing fetal lung development, perinatal nicotine exposure has been shown to increase total airway resistance, and decrease dynamic compliance of the respiratory system in second generation offspring in animal models.¹⁸

Nicotine exposure has also been related to abnormal placental structure and function. It has been shown to have an inhibitory effect on trophoblast migration, invasion and differentiation,^{19,20} alter the release of vasoactive factors by trophoblasts²¹ and also influence the transport of amino acids.²² Despite the large amount of evidence supporting the effect of IUS on the developing lung and the placental tissue, the molecular mechanisms have not been completely understood. One potential mechanism is DNA methylation, and a number of studies have examined DNA methylation changes in cord blood²³⁻²⁶ and placental tissue as a result of smoke exposure.²⁷⁻²⁹ Decreased DNA methyltransferase 1 (DNMT1) and increased DNA methyltransferase 3b

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(DNMT3b) expression have been shown to occur in human small airway smooth muscle cells exposed to cigarette smoke condensate.³⁰ In placental tissue, Suter et al. demonstrated the correlation between CYP1A1 expression and CpG methylation variation,³¹ thus supporting the role of DNA methylation. In another study, the authors assessed DNA methylation and gene expression in tobacco smoke exposed and control tissue at the genome wide level and found that there was a correlation between the 2 at a significantly higher number of genes in smoke-exposed tissue.²⁸ Global and gene specific changes have also been associated with in utero exposure in the buccal mucosa of children.³² Cord blood and placental samples obtained from pregnancies with gestational diabetes demonstrate *mesoderm specific transcript (MEST)* gene hypomethylation similar to that found in adults with morbid obesity,³³ thus suggesting that long-term disease programming occurring through DNA methylation may begin in utero. Studying these epigenetic patterns can allow us to understand and, potentially, interrupt disease development at an earlier stage.

Armstrong et al. recently studied DNA methylation at a global and gene specific level (7 genes) and found that placenta, cord blood and saliva varied in their DNA methylation patterns.³⁴ Similarly, other researchers have found that DNA methylation is tissue specific and that there are a small number of sites that share methylation patterns across tissues.³⁵ In this study, we investigated the variability of DNA methylation concurrently in lung tissue (which suffers the influence of this exposure) and the placenta (which may act as biomarker and mediator of exposure), to identify genes and disease pathways impacted by nicotine exposure.

Results

Demographic features of lung (n = 85, 41 smoke exposed, 44 controls) and placental samples (n = 80, 39 smoke exposed, 41 controls) that passed quality-control have been summarized (Table 1). We used placental cotinine as a marker of in utero nicotine exposure; the most likely source of nicotine is smoking and placental cotinine was previously shown to be a useful biomarker of IUS exposure. In a previous study, our group has demonstrated the relationship between placental cotinine levels and gene expression changes in fetal liver and lung.³⁶ Exposure to nicotine was dichotomized at a cotinine level of 7.5 ng/g placental tissue as this level has been shown to have a high sensitivity and specificity in identifying smoke exposure in pregnancy.³⁶

In the lung tissue, we analyzed the association of DNA methylation with nicotine exposure and found 264 CpG sites (68 CpG sites type I probes and 196 CpG sites type II probes) with an unadjusted *P*-value < 0.05 (see Table S4 and S5 in the online data supplement). CpG sites that had an association with a *P*-value < 0.01 after removal of sites likely to be influenced by SNPs, as described in the methods, have been listed with their mean β values in nicotine exposed and control tissue (Table 2). After adjusting for post-conceptual age, 226 sites remained significant at a nominal *P*-value < 0.05. None of the CpG sites in the lung tissue analysis remained significant after adjustment for multiple comparisons. The top CpG sites demonstrate both relative higher or lower methylation in nicotine exposed versus unexposed tissue, depending upon the CpG site evaluated (Figs. 1–2); thus, the directionality of association with nicotine varies from site to site.

In the placental tissue, 657 CpG sites had unadjusted nominal *P*-values < 0.05 (377 CpG sites type I and 280 sites type II probes). After adjustment for multiple comparisons and post-conceptual age, 2 sites had a Benjamini-Hochberg (BH) *P*-value < 0.05 and 6 sites had a *P*-value < 0.1 (see Table S6 and S7 in the online data supplement). A total of 469 CpG sites remained significant at a nominal *P*-value < 0.05 after adjusting for post-conceptual age. CpG sites with nominal *P* < 0.01 after removal of sites with SNPs have been listed in table 3. CpG sites that were most significant in the placental analyses mapped to the *GTF2H2C* and *GTF2H2D* genes on chromosome 5. Percent methylation in exposed and unexposed placental tissue for all CpG sites in the *GTF2H2C* and *GTF2H2D* gene suggest a region of relative hypomethylation associated with nicotine exposure (Fig. 3).

Nicotine likely has systemic molecular effects as well as tissue specific effects. To evaluate methylation differences that may not be tissue specific, we also assessed for sites that were concordant for the association with methylation in lung and placental analyses. One hundred and one CpG sites were found to overlap between the lung and placental analyses (see Table S8 in the online data supplement, Fig. 4a and b), and all these sites had the same directionality in both the tissues. Table 4 shows a subset of these overlapping sites, which were obtained after assessment for SNPs, associated with nicotine exposure in lung and placental tissue.

Pyrosequencing of selected sites from the lung and placental tissue was carried out on a smaller subset of samples and CpG sites, since we had limited DNA amounts remaining from the fetal tissues. For the lung samples, significant correlations between the illumina array percent methylation and pyrosequencing results were observed for *CNTD2* cg19605788, *DPP10*

Table 1 Demographic Features of Lung and Placental Samples

	Lung Tissue		Placental Tissue	
	Nicotine Exposed	Controls	Nicotine Exposed	Controls
Total Samples	41	44	39	41
Post Conception Age	57-115(85)	59-122(89)	57-113(85)	59-122(90)
Sex				
Males	24	28	22	24
Females	17	16	17	17
Mean Cotinine (ng/g placental tissue)	76.7 (11.5-186.4)	0.8 (0-5.2)	76.7 (11.5-186.4)	0.8 (0-5.2)

Table 2 Differential DNA methylation for single CpG sites and nicotine exposure for lung samples ($P < 0.01$)

NAME	CHR	Gene Symbol	logFC	P-value*	Mean β -value in controls [#]	Mean β in nicotine exposed [#]	logFC Age adjusted	P-value Age adjusted
cg23361828	2		-0.07	1.76E-03	0.61	0.54	-0.06	3.18E-03
cg17165241	11	<i>PKP3</i>	0.10	2.94E-03	0.63	0.72	0.09	3.71E-03
cg25612428	5	<i>ANKRD33B</i>	-0.07	3.12E-03	0.32	0.25	-0.07	5.42E-03
cg20142762	19	<i>NCR1</i>	0.07	3.67E-03	0.39	0.46	0.07	3.32E-03
cg19605788	19	<i>CNTD2</i>	0.06	4.90E-03	0.32	0.38	0.06	3.55E-03
cg20905796	11		0.09	4.93E-03	0.27	0.36	0.10	2.72E-03
cg22670147	2	<i>DPP10</i>	-0.06	5.43E-03	0.18	0.11	-0.07	3.14E-03
cg15864601	17	<i>C17orf97</i>	-0.07	5.91E-03	0.24	0.17	-0.07	3.45E-03
cg14223671	16	<i>PRR25</i>	-0.06	6.65E-03	0.29	0.22	-0.07	4.34E-03
cg07509935	14	<i>LTB4R2; CIDEB; LTB4R</i>	-0.08	6.71E-03	0.23	0.15	-0.08	9.07E-03
cg27055313	14		0.08	7.14E-03	0.34	0.42	0.08	1.19E-02
cg08537890	12		0.08	7.43E-03	0.43	0.51	0.08	9.41E-03
cg02407415	16	<i>PRR25</i>	-0.09	7.76E-03	0.33	0.24	-0.09	5.74E-03
cg06531573	16	<i>PRR25</i>	-0.08	8.04E-03	0.33	0.25	-0.08	5.68E-03
cg19430537	17		-0.10	9.13E-03	0.51	0.41	-0.08	2.38E-02
cg07234876	8		0.10	9.20E-03	0.71	0.81	0.10	1.76E-02

[#] Mean β -values are the raw means in nicotine exposed and unexposed (control) tissue.

* P-values for nicotine exposure coefficient from the limma model.

logFC: log fold change obtained from the limma model.

cg22670147, and *KLHL35* cg05353869 (Table 5). For the placental tissue, we observed significant correlations for *GTF2H2C* (cg02299136 and cg23813556) and *KLHL35* cg05353869 (Table 6). The pyrosequencing results for *PRR25* cg02407415 had the lowest correlations for lung and placenta (Pearson: 0.34 and 0.57, respectively). The CpG in *PRR25* site spans a region with a number of “A” homopolymers, which may partially account for these lower correlations.

KEGG pathway analysis was performed using gene set enrichment analysis (GSEA).³⁷ Two different pre-ranked lists of genes that were obtained using the absolute log fold change and the log fold change were used to assess enrichment. When using the first approach we found that the pathways obtained were similar in the lung and placental tissue, and included immune-related and asthma pathways (Table 7). When we used the second approach, none of the pathways were found to be significant at a

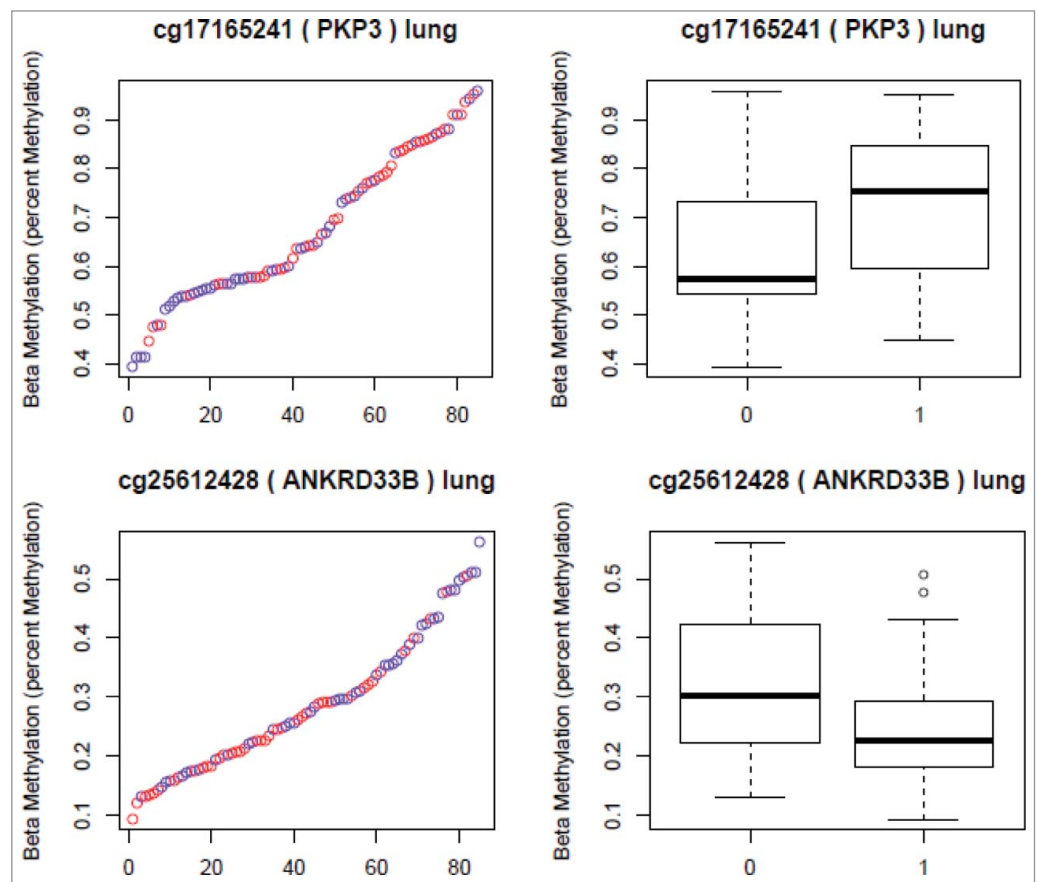


Figure 1. CpG sites Selected from the Lung Analyses. (Red in the scatter plots and 1 in the boxplots represents nicotine exposed tissue. Blue in the scatter plots and 0 in the boxplots represents unexposed tissue). The Y axis in both plots represents the β -values. The X axis in the scatter plots represents individual samples.

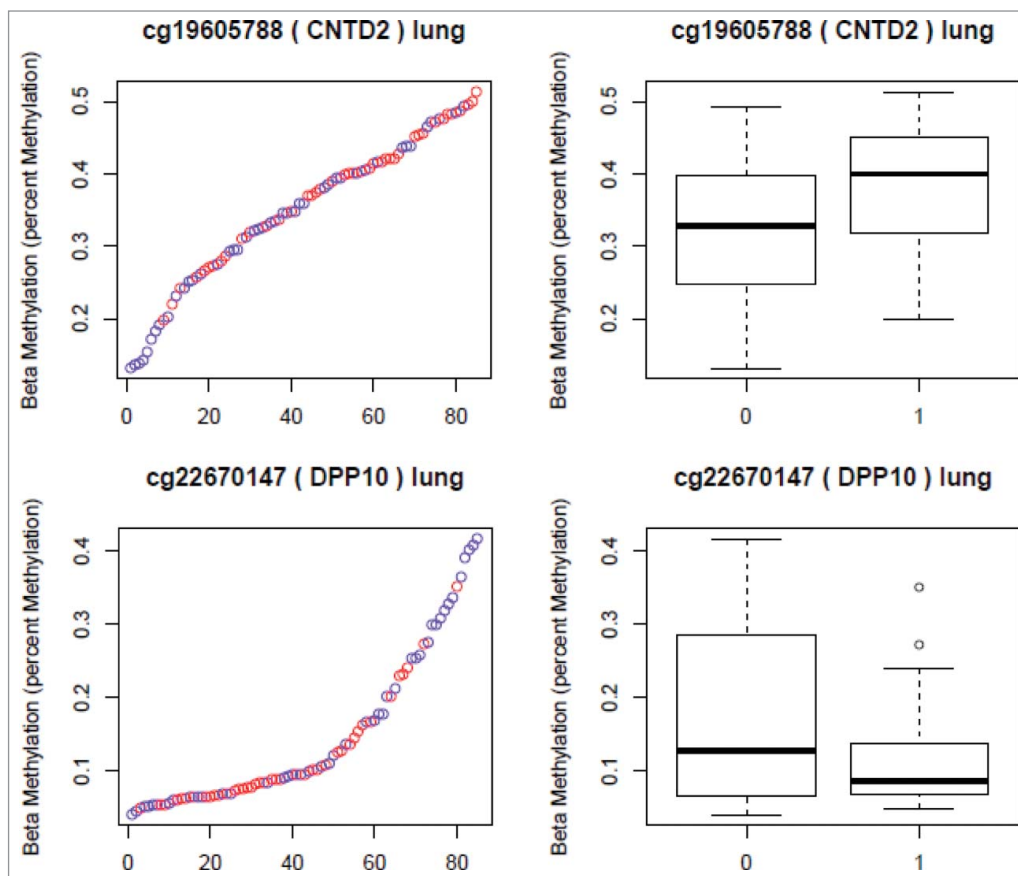


Figure 2. CpG sites Selected from the Lung Analyses. (Red in the scatter plots and 1 in the boxplots represents nicotine exposed tissue. Blue in the scatter plots and 0 in the boxplots represents unexposed tissue). The Y axis in both plots represents the β -values. The X axis in the scatter plots represents individual samples.

studied genome-wide methylation in matched lung and placental tissue. To our knowledge, this is the first time methylation in developing human fetal lung tissue and placental tissues have been assessed together. The overlap of pathways between the placenta and the lung is one more piece of evidence that the effects of in utero exposure are likely to be enduring and potentially systemic and have long-term effects on the developing fetus and subsequent risk for chronic lung diseases such as asthma.

One of the genes most significantly associated with DNA methylation changes in the lung was *DPP10*, which has been associated with asthma in a number of studies.⁴⁴⁻⁴⁶ Thus, it is plausible that methylation variation as a result of smoke exposure predisposes an infant to develop lung disease later in life through epigenetic disruption of key developmental

false discovery rate (FDR) of 0.05. Pathways with a nominal P -value < 0.05 have been listed in the online data supplement (Table S9 and S10).

Variable DNA methylation at a number of different genes has previously been associated with IUS exposure. As such, we specifically assessed CpG sites in the *FRMD4A*, *C11orf52*, *XPNPEP1*, *PPEF2*, *SMPD3*, *CRYGN*,³⁸ *TSLP*,²⁶ *IGF2*,²³ *PURA*, *GTF2H2*, *GCA*, *GPR135*, *HKR1*,²⁸ *AXL*,^{32, 39} *BDNF*,⁴⁰ *PTPRO*,³² *HSD11B*,²⁹ *NR3C1*,⁴¹ *AHRR*, *CYP1A1*, *GFI1*,²⁴ *GSTM5*, *MAP2K3*, and *APOB*⁴² genes in lung and placental tissue. The only sites that were noted to be significant after adjusting for multiple comparisons mapped to the *GTF2H2C*, *GTF2H2D* region, which was added to the search, as no CpG sites mapped to the *GTF2H2* gene (Table S11A, S11B, S12A and S12B).

Discussion

We identified significant pathway-related differential methylation associated with nicotine exposure, including evidence suggesting that asthma and other immune pathways may be perturbed by this exposure. To investigate the “molecular archive”⁴³ associated with in utero exposure to nicotine, we

and homeostatic pathways; further research in human cohorts and animal models is warranted. Epidemiological data support the influence of in-utero exposures on long-term outcomes and alteration in DNA methylation is one potential mechanism through which environmental exposures can impact human health and disease. Experimental evidence suggests that methylation changes occurring during development are tissue specific and play a role in tissue differentiation.⁴⁷ A number of studies on placental tissue have demonstrated variable methylation in the setting of preeclampsia,^{48,49} pregnancies conceived by assisted reproductive technologies,⁵⁰ gestational diabetes,³³ and maternal exposures such as smoking.²⁷⁻²⁹ Variation in DNA methylation associated with cigarette smoking has also been demonstrated in studies of DNA from peripheral blood leukocytes,⁵¹ peripheral blood,⁵² lymphoblasts, and alveolar macrophages⁵³ in adults. Studies in neonates have shown IUS exposure associated with variation in methylation of DNA in umbilical cord blood²³⁻²⁶ and placental tissue.²⁷⁻²⁹ Other studies have shown an association of prenatal smoke exposure with DNA methylation in buccal source DNA in children,³² blood in adolescents,⁴⁰ and peripheral blood granulocytes in adult women.⁵⁴ The potential health-related relevance of epigenetic variability in placental tissue on clinical outcomes has been suggested by a study which showed

Table 3 Differential methylation for single CpG sites and nicotine exposure for placental samples ($P < 0.01$)

NAME	CHR	Gene Symbol	logFC	P-value*	Mean β -value in controls [#]	Mean β -value in smoke exposed [#]	BH P-value	logFC (Age adjusted)	P-value (Age adjusted)	BH P-value after adjusting for age
cg27324075	5	<i>GTF2H2C; GTF2H2D</i>	-0.14	2.87E-06	0.23	0.09	3.56E-02	-0.14	2.78E-06	3.41E-02
cg17290897	5	<i>GTF2H2C; GTF2H2D</i>	-0.13	5.92E-06	0.19	0.06	3.66E-02	-0.13	5.51E-06	3.41E-02
cg06966320	5	<i>GTF2H2C; GTF2H2D</i>	-0.10	2.11E-05	0.15	0.05	7.70E-02	-0.10	2.04E-05	7.12E-02
cg02299136	5	<i>GTF2H2C; GTF2H2D</i>	-0.10	2.49E-05	0.21	0.10	7.70E-02	-0.11	2.30E-05	7.12E-02
cg23813556	5	<i>GTF2H2C; GTF2H2D</i>	-0.11	3.48E-05	0.24	0.13	7.70E-02	-0.11	3.57E-05	8.84E-02
cg07730364	1	<i>KIAA1614</i>	0.11	3.73E-05	0.12	0.23	7.70E-02	0.10	9.24E-05	1.91E-01
cg23056823	1	<i>KIAA1614</i>	0.12	6.24E-05	0.08	0.20	1.10E-01	0.12	1.14E-04	2.01E-01
cg27262412	1	<i>TBX15</i>	0.09	1.36E-04	0.36	0.45	2.11E-01	0.08	3.84E-04	4.76E-01
cg08305436	19		0.10	1.54E-04	0.26	0.36	2.13E-01	0.10	2.27E-04	3.51E-01
cg21392341	1	<i>TBX15</i>	0.09	6.04E-04	0.31	0.40	6.23E-01	0.08	1.45E-03	1.00E+00
cg03712062	4		0.08	7.35E-04	0.49	0.57	7.00E-01	0.07	1.63E-03	1.00E+00
cg17038216	12		0.08	9.36E-04	0.55	0.63	8.28E-01	0.08	1.73E-03	1.00E+00
cg00197191	16		0.08	1.08E-03	0.56	0.64	8.94E-01	0.07	2.28E-03	1.00E+00
cg07029873	1	<i>ESRRG</i>	0.08	1.43E-03	0.42	0.50	9.26E-01	0.07	3.69E-03	1.00E+00
cg10516465	14	<i>RAGE</i>	0.08	1.57E-03	0.58	0.66	9.26E-01	0.09	3.76E-04	4.76E-01
cg08801017	13	<i>LOC100101938</i>	0.07	1.83E-03	0.31	0.38	9.26E-01	0.07	1.82E-03	1.00E+00
cg19178853	4	<i>NBLA00301; HAND2</i>	0.10	2.49E-03	0.23	0.33	9.26E-01	0.10	3.22E-03	1.00E+00
cg01189072	1	<i>CDK11B; LOC728661</i>	-0.08	2.67E-03	0.90	0.82	9.26E-01	-0.08	3.14E-03	1.00E+00
cg16875032	8	<i>SNTB1</i>	0.08	2.85E-03	0.12	0.19	9.26E-01	0.07	5.46E-03	1.00E+00
cg18802021	10	<i>MKX</i>	0.09	2.94E-03	0.13	0.22	9.26E-01	0.07	8.57E-03	1.00E+00
cg26121931	6		0.09	3.01E-03	0.27	0.35	9.26E-01	0.09	4.32E-03	1.00E+00
cg04741284	20		0.07	3.36E-03	0.26	0.33	9.26E-01	0.05	1.06E-02	1.00E+00
cg22728830	11		0.07	3.46E-03	0.44	0.51	9.26E-01	0.07	6.32E-03	1.00E+00
cg09264282	1	<i>COL24A1</i>	0.08	3.49E-03	0.23	0.31	9.26E-01	0.07	5.93E-03	1.00E+00
cg04550737	1	<i>TBX15</i>	0.10	3.74E-03	0.34	0.43	9.26E-01	0.09	9.27E-03	1.00E+00
cg24603464	2	<i>MLPH</i>	0.09	3.79E-03	0.33	0.43	9.26E-01	0.08	9.74E-03	1.00E+00
cg05133150	20	<i>SPINT3</i>	0.07	4.09E-03	0.60	0.67	9.26E-01	0.07	3.42E-03	1.00E+00
cg04866628	4		0.07	4.11E-03	0.27	0.34	9.26E-01	0.06	9.51E-03	1.00E+00
cg09676860	2	<i>RAB6C</i>	0.07	4.14E-03	0.25	0.32	9.26E-01	0.06	7.88E-03	1.00E+00
cg09489306	19		-0.08	4.19E-03	0.45	0.37	9.26E-01	-0.08	2.21E-03	1.00E+00
cg15309006	16	<i>CHP2</i>	0.07	4.40E-03	0.20	0.27	9.26E-01	0.06	1.33E-02	1.00E+00
cg16043651	19	<i>ZNF536</i>	0.08	4.66E-03	0.36	0.44	9.26E-01	0.07	7.52E-03	1.00E+00
cg22474464	20	<i>NKX2-2</i>	0.08	4.67E-03	0.19	0.27	9.26E-01	0.07	1.18E-02	1.00E+00
cg14709460	7	<i>TRIL</i>	0.07	4.69E-03	0.14	0.20	9.26E-01	0.06	8.33E-03	1.00E+00
cg08315202	7	<i>NPTX2</i>	0.08	4.71E-03	0.16	0.23	9.26E-01	0.07	1.06E-02	1.00E+00
cg08581512	11	<i>CYP2R1</i>	0.08	4.76E-03	0.15	0.22	9.26E-01	0.07	1.27E-02	1.00E+00
cg25594899	1	<i>FAM132A</i>	-0.07	4.98E-03	0.17	0.10	9.26E-01	-0.07	6.45E-03	1.00E+00
cg09798888	12		0.07	5.14E-03	0.36	0.44	9.26E-01	0.06	1.22E-02	1.00E+00
cg15487867	1	<i>C1orf70</i>	0.08	5.23E-03	0.33	0.41	9.26E-01	0.08	8.46E-03	1.00E+00
cg09373037	10	<i>SYT15</i>	0.07	5.31E-03	0.16	0.22	9.26E-01	0.06	1.42E-02	1.00E+00
cg07176264	2	<i>SCTR</i>	0.08	5.33E-03	0.16	0.25	9.26E-01	0.07	1.55E-02	1.00E+00
cg15684724	8		0.07	5.50E-03	0.15	0.22	9.26E-01	0.07	1.17E-02	1.00E+00
cg17073273	6		0.06	5.98E-03	0.27	0.33	9.26E-01	0.05	1.80E-02	1.00E+00
cg23697417	20	<i>RSPO4</i>	0.08	6.00E-03	0.20	0.28	9.26E-01	0.06	2.00E-02	1.00E+00
cg05932159	6	<i>HLA-B</i>	0.07	6.18E-03	0.25	0.32	9.26E-01	0.06	1.92E-02	1.00E+00
cg02329935	5	<i>PDE8B</i>	0.07	6.32E-03	0.25	0.32	9.26E-01	0.06	1.39E-02	1.00E+00
cg24475782	1	<i>TBX15</i>	0.06	6.46E-03	0.40	0.47	9.26E-01	0.05	1.74E-02	1.00E+00
cg11823511	1	<i>BARHL2</i>	0.07	6.67E-03	0.12	0.19	9.26E-01	0.07	1.46E-02	1.00E+00
cg25464210	19	<i>ZNF85</i>	0.06	6.82E-03	0.12	0.18	9.26E-01	0.06	1.20E-02	1.00E+00
cg06970200	10		0.07	6.98E-03	0.67	0.74	9.26E-01	0.06	1.39E-02	1.00E+00
cg01419567	8	<i>ZFHX4; LOC100192378</i>	0.06	6.99E-03	0.09	0.15	9.26E-01	0.06	1.26E-02	1.00E+00
cg16601494	1	<i>C1orf70</i>	0.07	7.21E-03	0.45	0.52	9.26E-01	0.07	1.08E-02	1.00E+00
cg19970425	15	<i>RAB27A</i>	-0.09	7.43E-03	0.22	0.13	9.26E-01	-0.09	5.97E-03	1.00E+00
cg13849454	9		0.07	7.59E-03	0.32	0.39	9.26E-01	0.06	1.87E-02	1.00E+00
cg25035631	1		0.07	7.69E-03	0.27	0.33	9.26E-01	0.06	1.67E-02	1.00E+00
cg25284624	1		-0.07	7.72E-03	0.31	0.24	9.26E-01	-0.07	1.11E-02	1.00E+00
cg18197392	20	<i>SSTR4</i>	0.06	7.94E-03	0.18	0.24	9.26E-01	0.06	1.30E-02	1.00E+00
cg11276420	11	<i>GRIA4</i>	0.07	7.95E-03	0.57	0.64	9.26E-01	0.07	4.84E-03	1.00E+00
cg21442271	22		0.09	8.00E-03	0.48	0.58	9.26E-01	0.10	3.59E-03	1.00E+00

(continued on next page)

Table 3 Differential methylation for single CpG sites and nicotine exposure for placental samples ($P < 0.01$) (Continued)

NAME	CHR	Gene Symbol	logFC	P-value*	Mean β -value in controls [#]	Mean β -value in smoke exposed [#]	BH P-value	logFC (Age adjusted)	P-value (Age adjusted)	BH P-value after adjusting for age
cg01558909	16	HBM	0.07	8.03E-03	0.09	0.16	9.26E-01	0.06	1.97E-02	1.00E+00
cg18433519	6	KIAA0319	-0.11	8.08E-03	0.32	0.21	9.26E-01	-0.10	1.39E-02	1.00E+00
cg12571892	4	PDE5A	0.06	8.27E-03	0.52	0.58	9.26E-01	0.07	7.66E-03	1.00E+00
cg16565890	6	RIMS1	0.07	8.30E-03	0.54	0.61	9.26E-01	0.07	1.03E-02	1.00E+00
cg14004073	5	FAM159B	0.07	8.34E-03	0.18	0.25	9.26E-01	0.06	1.79E-02	1.00E+00
cg27170947	2	FAM59B	0.07	8.44E-03	0.17	0.24	9.26E-01	0.06	1.64E-02	1.00E+00
cg13946520	2	CD8A	0.06	8.56E-03	0.17	0.23	9.26E-01	0.05	2.38E-02	1.00E+00
cg10541864	4	HAND2; NBLA00301	0.09	8.62E-03	0.36	0.45	9.26E-01	0.09	1.29E-02	1.00E+00
cg21448033	19	ZNF85	0.07	8.66E-03	0.14	0.21	9.26E-01	0.07	1.54E-02	1.00E+00
cg21913319	1	COL24A1	0.11	8.70E-03	0.28	0.40	9.26E-01	0.11	1.47E-02	1.00E+00
cg07657192	6		0.06	8.84E-03	0.12	0.18	9.26E-01	0.05	2.24E-02	1.00E+00
cg12200038	14		0.06	8.89E-03	0.29	0.36	9.26E-01	0.05	2.13E-02	1.00E+00
cg15074838	6	HLA-DRA	0.06	9.09E-03	0.46	0.52	9.26E-01	0.06	1.31E-02	1.00E+00
cg06414921	6		0.07	9.20E-03	0.22	0.30	9.26E-01	0.07	1.91E-02	1.00E+00
cg11471802	8		0.07	9.35E-03	0.40	0.47	9.26E-01	0.08	9.34E-03	1.00E+00
cg06204629	1		0.06	9.44E-03	0.57	0.63	9.26E-01	0.07	6.85E-03	1.00E+00
cg25036284	2	FAM59B	0.09	9.65E-03	0.17	0.26	9.26E-01	0.09	1.66E-02	1.00E+00
cg22725685	4		0.08	9.69E-03	0.26	0.34	9.26E-01	0.08	1.41E-02	1.00E+00

[#] Mean β -values are the raw means in nicotine exposed and unexposed (control) tissue.

* P-values for nicotine exposure coefficient from the limma model.

logFC: log fold change obtained from the limma model.

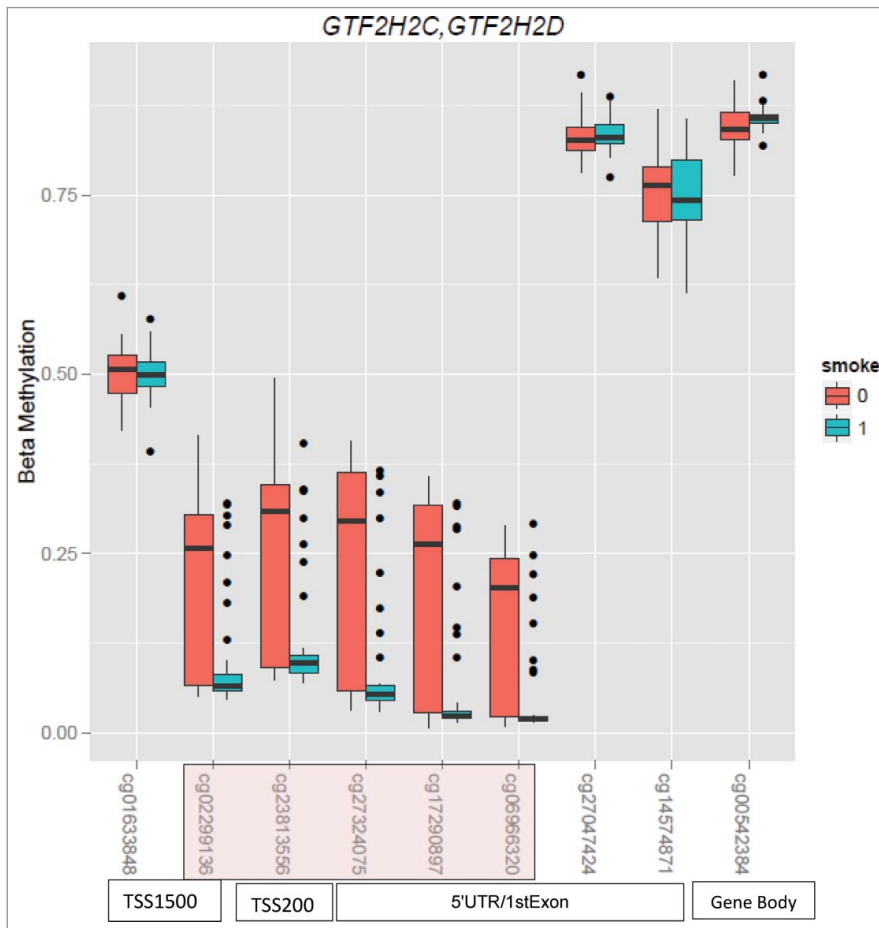


Figure 3. Boxplots of CpG sites in the *GTF2H2C/GTF2H2D* gene in placental tissue. Sites highlighted in pink represents sites that have a BH adjusted P -value < 0.1 .

that methylation status of *11- β hydroxysteroid dehydrogenase* was associated with infant quality of movement scores.⁵⁵ Similarly, birth weight has been associated with placental *glucocorticoid receptor* gene methylation.⁵⁶ Thus, investigating epigenetic variability in fetal tissues as a “molecular archive” of the prenatal environmental⁴³ provides an opportunity to identify and potentially intervene at an early stage in disease trajectories.

The top CpG sites in placental tissue mapped to the *general transcription factor IIIH, polypeptide 2C (GTF2H2C)* and *general transcription factor IIIH, polypeptide 2D (GTF2H2D)* genes. In an epigenome-wide analysis carried out on placental DNA from term pregnancies using the Illumina HumanMethylation27 BeadChip array, Suter et al.²⁸ reported a difference of more than 10% in placental methylation of *GTF2H2* between smokers and non-smokers. Based on similarity, general transcription factor IIIH subunit 2-like protein is believed to be a component of the core-TFIID basal transcription factor, which plays a role in DNA nucleotide excision repair and transcription by RNA polymerase II.⁵⁷ Transcription factor IIIH has been shown to have a significantly lower expression in alveolar macrophages in subjects with idiopathic pulmonary fibrosis.⁵⁸ Thus,

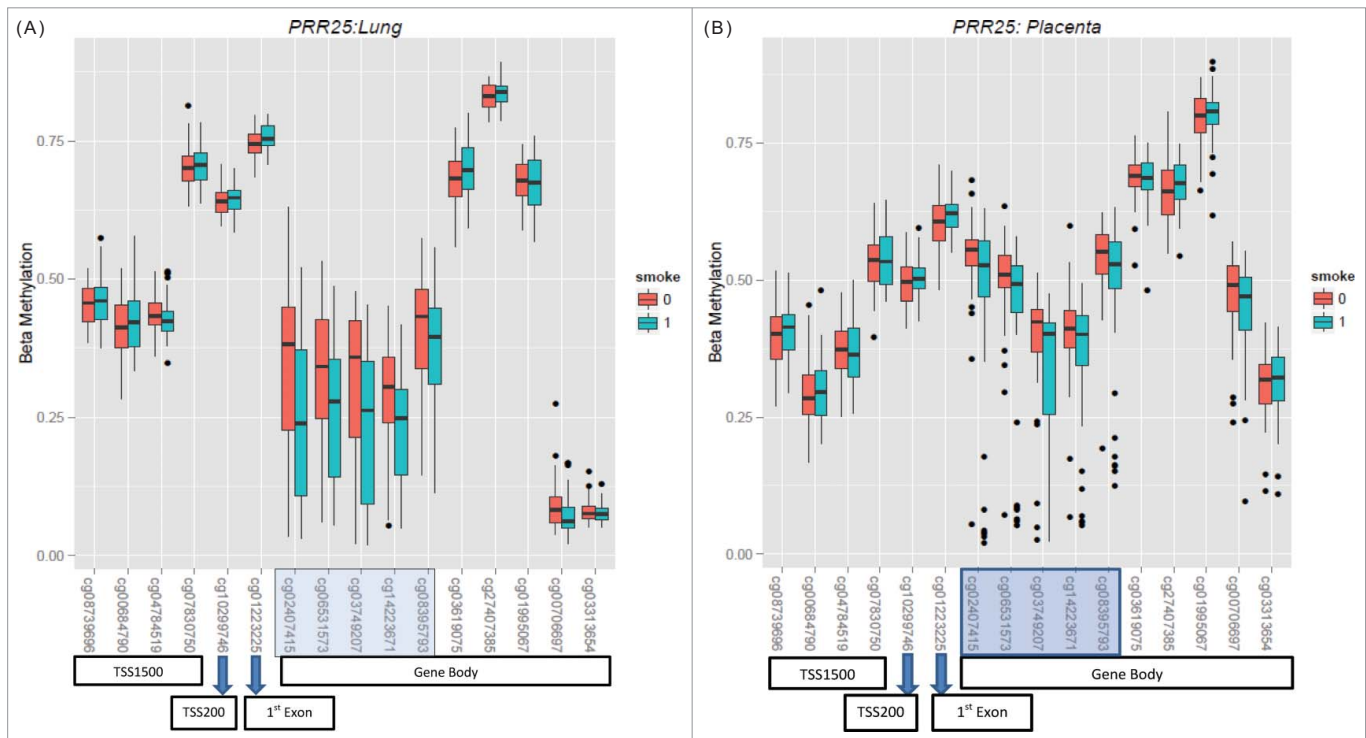


Figure 4. (A). Boxplots of CpG sites in the *PRR25* gene in lung tissue. Sites highlighted in blue represents sites that have a nominal *P*-value < 0.1. (B). Boxplots of CpG sites in the *PRR25* gene in placental tissue. Sites highlighted in blue represent those that have a nominal *P*-value < 0.1.

Table 4 Overlapping CpG sites in the lung and placental analyses

NAME	CHR	Gene Symbol	Difference in mean β -values: Lung (exposed-unexposed) [#]	Difference in mean β -values: Placenta (exposed-unexposed) [#]
cg12036633	15		0.16	0.15
cg21442271	22		0.11	0.09
cg11478607	22	<i>GSTT1</i>	0.10	0.08
cg16875032	8	<i>SNTB1</i>	0.06	0.08
cg08801017	13	<i>LOC100101938</i>	0.05	0.07
cg10909185	11	<i>KLHL35</i>	0.08	0.07
cg05353869	11	<i>KLHL35</i>	0.07	0.06
cg27055313	14		0.08	0.06
cg10546252	22	<i>GSTT1</i>	0.09	0.06
cg05840289	5	<i>ANKRD33B</i>	0.06	0.06
cg02405213	9	<i>JAK2</i>	0.06	0.06
cg08537890	12		0.08	0.05
cg19596870	6	<i>SERPIN6</i>	0.09	0.05
cg08845336	6	<i>HLA-DRB1</i>	-0.08	-0.06
cg03749207	16	<i>PRR25</i>	-0.08	-0.06
cg06531573	16	<i>PRR25</i>	-0.08	-0.06
cg01826367	1		-0.05	-0.07
cg02407415	16	<i>PRR25</i>	-0.09	-0.09
cg18433519	6	<i>KIAA0319</i>	-0.10	-0.11

[#] Mean β -values are the raw means in nicotine exposed and control tissue. Sites listed were found to be significant at a nominal *P*-value of 0.05, in both lung and placental tissue analysis, after removal of sites with SNPs or apparent SNP effects.

altered methylation in *GTF2H2C* and *GTF2H2D* may occur in an attempt to repair the DNA damage resulting from exposure to cigarette smoke.

Among the overlapping sites in the lung and placental analyses was the *Janus Kinase 2 (JAK2)* gene, which encodes a protein tyrosine kinase that plays a role in a number of pathways, including cell growth, development, differentiation, and histone modifications.⁵⁹ *JAK2* is also involved in erythropoietin signal transduction and mutations in this genes have been associated with polycythemia vera⁶⁰⁻⁶³ and other myeloproliferative disorders.^{63,64} Though it was not associated with a higher hematocrit, the *JAK2* mutation was found more frequently in smokers than in non-smokers.⁶⁵ In a study of non-smokers with bladder carcinomas, increased DNA methylation of the *JAK2* gene was associated with total and occupational smoke exposure,⁶⁶ but was not associated with childhood exposure. In our analysis, we found this locus to be hypermethylated in nicotine-exposed lung and placental tissue. Thus, variable methylation may be an intermediate state resulting from smoke exposure.

We acknowledge several potential limitations to our study, including the paucity of clinical/demographic data on our samples. We are considering the source of elevated placental cotinine to be IUS. However, as cotinine is a metabolite of nicotine metabolism, the mothers could have been exposed to nicotine in other forms besides active smoking, including passive smoking, e-cigarette use, and nicotine replacement therapy. As these are

Table 5 Pyrosequencing results: Lung

	n	Pearson Correlation Coefficient	P-value	Spearman Correlation Coefficient	P-value
<i>CNTD2</i> (cg19605788)	27	0.57	1.71E-03	0.58	1.58E-03
<i>DPP10</i> (cg22670147)	31	0.73	2.75E-06	0.65	6.49E-05
<i>KLHL35</i> (cg05353869)	30	0.84	8.11E-09	0.80	9.86E-08
<i>PRR25</i> (cg02407415)	29	0.34	6.90E-02	0.41	2.63E-02

anonymized samples, we were unable to assess effects of racial distribution on variable methylation. We did apply the approach of Barfield et al.⁶⁷ to explore population substructure; however, given the relatively small size of our study, we present the adjusted *P*-values for the top sites in the supplemental data. It is not known if there were any underlying pathologies, nor could we adjust for other maternal prenatal behaviors, such as alcohol ingestion, use of other drugs or medications, or quality of routine prenatal care. We suggest that stringent quality control steps and removal of outlier samples would mitigate the influence of any such effect. Surrogate variable analysis also showed the absence of batch effects in our unadjusted model. The lack of CpG sites that were significantly altered in lung tissue analysis at an adjusted *P*-value of 0.05 is likely a function of our sample size. However, this observation is offset by the statistically robust and biologically plausible results of our pathway analysis. Lastly, we have been able to perform technical validation, using pyrosequencing, only on a small subset of CpG sites and samples due to the limited DNA availability from these precious fetal lung tissue samples. Future studies that focus on larger sample sizes could help further support the impact of IUS in lung and placental tissue, as well as address issues related to population substructure.

We conclude that variable methylation is a potential marker of injury in the developing lung exposed to nicotine in utero. In addition, a subset of methylation marks perturbed by nicotine exposure is recapitulated in the placenta. The enrichment in asthma and immune pathway genes observed in our analyses raises the possibility that variable methylation may be used as a biomarker of future risk of disease during childhood or adult life. Longitudinal studies and replication in other cohorts would add to our knowledge of lung diseases and other chronic illnesses resulting from nicotine/IUS exposure. Although the public health message remains that all individuals, especially pregnant women, should not smoke cigarettes (the most likely source of elevated placental cotinine), our research lays the groundwork for the identification of an epigenetic signature that could inform primary prevention and early diagnosis of chronic human diseases associated with IUS and in utero nicotine exposure.

Table 6 Pyrosequencing results: Placenta

	n	Pearson Correlation Coefficient	P-value	Spearman Correlation Coefficient	P-value
<i>GTF2H2C</i> (cg02299136)	42	0.83	9.18E-12	0.73	4.39E-08
<i>GTF2H2C</i> (cg23813556)	42	0.80	3.11E-10	0.70	8.77E-07
<i>KLHL35</i> (cg05353869)	41	0.82	4.46E-11	0.74	4.13E-08
<i>PRR25</i> (cg02407415)	36	0.57	2.65E-04	0.45	5.81E-03

Patients and Methods

Anonymized fetal lung and placental tissue samples were acquired through the NICHD-supported tissue retrieval program at the Laboratory of Developmental Biology, University of Washington, Seattle, WA. These tissues have been designated as non-human subjects by the Children's Mercy Hospital Pediatric Institutional Review Board. Phenotypic information on these samples is limited to post-conception age. Nicotine exposure was determined by measuring placental cotinine concentration in nanograms per gram of placental tissue. Exposure was treated as a dichotomous variable, with levels of cotinine < 7.5 ng/g being considered as unexposed (control group) and levels of cotinine > 7.5 ng/g as exposed. This level has been previously shown to have a sensitivity of 78.7% and a specificity of 100% for identifying mothers that smoked during pregnancy.³⁶ However, given the limited clinical history of the mothers, the actual source of positive nicotine levels is undetermined. Post-conceptual age was assessed based on the foot length of the fetus.

Placental and lung tissues were flash frozen at the time of procurement and stored at -80°C. Lung tissue and placental DNA from 95 subjects were quantified in our laboratory using standard pico-green methods. Genome-wide methylation assay was performed with 750 ng of bisulfite-treated DNA per sample using the Illumina HumanMethylation450 BeadChip array, according to manufacturer's recommended protocol. Placental and lung tissue samples from the same subject were spotted on the same array. Results were extracted using GenomeStudio (Software v2011.1) and then read into Bioconductor (version 2.12) for further analyses. Percent methylation was reported as the Illumina β -value, which is the ratio between methylated signal intensity and total probe signal intensity.⁶⁸ Quality control steps performed for both lung and placental tissue DNA methylation marks have been described in the Supplemental data (see description and **Table S3** in the online data supplement). The Illumina Infinium HumanMethylation450 Bead Chip array has 135501 type I probes and 350076 type II probes that differ in assay chemistry and, thus, in the distribution of methylation values. It has been shown that the β -values (percent methylation) obtained from the Infinium type II probes have a smaller range when

Table 7 GSEA Analysis

NAME	SIZE	ES	NES	NOM <i>P</i> -value	FDR <i>q</i> -value
Lung Analysis					
Leishmania Infection	20	0.67	1.71	1.36E-03	2.17E-02
Type I Diabetes Mellitus	21	0.67	1.69	6.72E-04	2.34E-02
Allograft Rejection	21	0.64	1.62	2.70E-03	2.82E-02
Systemic Lupus Erythematosus	22	0.64	1.63	2.01E-03	2.93E-02
Autoimmune Thyroid Disease	24	0.66	1.72	0.00E+00	3.13E-02
Haematopoietic Cell Lineage	13	0.69	1.63	6.19E-03	3.40E-02
Graft vs. Host Disease	21	0.63	1.60	2.68E-03	3.48E-02
Intestinal Immune Network For IgA Production	17	0.71	1.75	1.36E-03	3.90E-02
Asthma	12	0.71	1.64	2.07E-03	3.91E-02
Placental Analysis					
Graft versus Host Disease	20	0.69	1.79	0.00E+00	3.65E-03
Allograft Rejection	19	0.71	1.80	1.34E-03	4.87E-03
Autoimmune Thyroid Disease	23	0.69	1.80	0.00E+00	6.97E-03
Antigen Processing and Presentation	24	0.70	1.82	0.00E+00	1.06E-02
Leishmania Infection	19	0.67	1.73	2.01E-03	1.11E-02
Type I Diabetes Mellitus	23	0.66	1.72	0.00E+00	1.20E-02
Cell Adhesion Molecules CAMS	42	0.60	1.67	0.00E+00	1.98E-02
Asthma	11	0.71	1.66	4.85E-03	2.19E-02
Viral Myocarditis	28	0.61	1.61	0.00E+00	3.83E-02

compared with those from Infinium type I probes.⁶⁹ Currently, there is no consensus in the literature on the best approach for normalization of the data in the presence of this diversity. For this analysis, quantile normalization and differential methylation analysis were performed separately on the Infinium type I and type II probes, using the *methylumi* package.⁷⁰ Type I probes were color adjusted prior to normalization. A large number of CpG sites in the genome showed very little variance. Therefore, only sites with a variance in percent methylation higher than 1% (1986 and 4737 CpG sites for lung tissue and 6938 and 5444 for placental tissue, for type I and II probes, respectively) were carried forward for analysis. Surrogate variable analysis was also performed to assess for batch effects in both fetal lung and placental data sets.⁷¹

Statistical analyses

Differential methylation analyses were carried out using linear models and empirical Bayes methods. These analyses were run using the Bioconductor package *limma*⁷² and results were considered together. For the initial analysis of the association of nicotine exposure—most likely a result of maternal cigarette smoke exposure—and methylation, we performed an unadjusted differential methylation analysis. The surrogate variable analysis for the unadjusted model was found to have zero surrogate variables in lung and placental tissue. Thereafter, *P*-values obtained for the type I and type II analyses were combined and adjusted for multiple comparisons using BH correction for multiple comparisons. We also carried out the *limma* analysis adjusting for post-conceptual age. CpG sites were mapped to genes using annotation provided by Illumina and, for instances in which a site mapped to more than one gene, the first gene name was randomly selected for pathway analysis. KEGG analysis of the pre-ranked list was performed using the gene set enrichment analysis (GSEA

v2.0.14). Two different approaches that involved ranking genes based on their log fold change and the absolute log fold change were used for GSEA. When ranking genes by absolute log fold change, the highest value of this parameter was chosen to determine the rank of genes with multiple CpG sites. However, when using the log fold change, we used the lowest *P*-value to determine the rank of genes for GSEA. For the lung analysis, 2981 genes were added to the GSEA analysis; for the placental tissue analysis, 4229 genes were added. The manually curated KEGG gene set (c2.cp.kegg.v4.0.symbols.gmt) from the Molecular Signatures Database (v4.0 MSigDB) was used as a reference set for the pathway analysis^{37,73}.

For CpG sites that were found to be significant in the *limma* analysis, we removed results in which a SNP was at a single base extension site or at the proximal CpG on the probe, using data obtained from the Bioconductor package *minfi*.⁷⁴ We also assessed scatter plots of methylation and removed sites with apparent SNP effects based on clustering of methylation values. Pyrosequencing of a limited number of sites that were selected based on significance in our analysis, absence of SNPs, having more than one significant CpG sites in the same genomic region, and biological plausibility was performed using a subset of lung and placental tissues (for which we had adequate DNA), as detailed in the Supplemental Methods. All probes used for pyrosequencing have been listed in the Supplemental Methods. After removal of failed samples and outliers, Pearson and Spearman correlations were assessed between the pyrosequencing results and the β -values used in our analysis. Literature review was carried out to obtain a list of genes for which DNA methylation had been previously observed as a result of IUS exposure. These sites were assessed in lung and placental tissue to determine if they were differentially methylated.

Disclosure of Potential Conflicts of Interest:

No conflicts of interest were disclosed.

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Contributor’s Statement

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Kelan G Tantisira: conception and design, acquisition of data, analysis and interpretation of data, review of manuscript and approval of the final version of the manuscript.

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher’s website.

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