Maternal tobacco smoke exposure is associated with increased DNA methylation at human metastable epialleles in infant cord blood

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Abstract
Metastable epialleles (MEs) are genomic regions that are stochastically methylated prior to germ layer specification and exhibit high interindividual but low intra-individual variability across tissues. ME methylation is vulnerable to environmental stressors, including diet. Tobacco smoke (TS) exposure during pregnancy is associated with adverse impacts on fetal health and maternal micronutrient levels as well as altered methylation. Our objective was to determine if maternal smoke exposure impacts methylation at MEs. Consistent with prior studies, we observed reductions in one-carbon pathway micronutrients with gestational TS exposure, including maternal folate (P = 0.02) and vitamins B6 (P = 0.05) and B12 (P = 0.007). We examined putative MEs BOLA3, PAX8, and ZFYVE28 in cord blood specimens from 85 Newborn Epigenetics Study participants. Gestational TS exposure was associated with elevated DNA methylation at PAX8 (+ 5.22% average methylation; 95% CI: 0.33% to 10.10%; P = 0.037). In human conceptus kidney tissues, higher PAX8 transcription was associated with lower methylation (R2 = 0.55; P = 0.07), suggesting that the methylation levels established at MEs, and their environmentally induced perturbation, may have meaningful, tissue-specific functional consequences. This may be particularly important because PAX8 is implicated in several cancers, including pediatric kidney cancer. Our data are the first to indicate vulnerability of human ME methylation establishment to TS exposure, with a general trend of increasing levels of methylation at these loci. Further investigation is needed to determine how TS exposure-mediated changes in DNA methylation at MEs, and consequent expression levels, might affect smoking-related disease risk.

Key words: metastable epiallele; DNA methylation; gestational tobacco smoke exposure; gene expression; umbilical cord blood; children’s health

Introduction
Metastable epialleles (MEs) were first identified in mammals in 2002 as regions of the genome that are expressed variably in genetically identical individuals [1] as a result of epigenetic modifications, particularly DNA methylation [2]. Methylation is stochastically established at these loci during development. The term “epiallele” refers to an allele that can exist in multiple epigenetic states. “Metastable” refers to the variable establishment of epigenetic marks, like DNA methylation, at epialleles during early development, before the specification of the germ layers [1]. Once established, the methylation level at MEs is somatically heritable and stable across the life course. As such, ME methylation patterns are consistent in all tissue types derived from the mesoderm, endoderm, and ectoderm of the same individual. However, due to the randomly determined nature of the level of initial methylation establishment, MEs are also characterized by strong interindividual variation [1, 3–6].

The developmental window during which methylation levels at mammalian MEs are established is particularly vulnerable to environmental stressors [3–9]. Recently, multiple MEs were identified in humans [6] and found to exhibit methylation patterns that were associated with nutritional challenges faced by the mother during the periconceptional period [5], in agreement with prior rodent studies [3, 4]. However, little is known about the vulnerability of human MEs to non-nutritional exposures, including the impact of tobacco smoke (TS) exposure during pregnancy.

Developmental TS exposure has been extensively characterized as a serious risk to fetal and infant health [10–12]. Epidemiological studies have found that smoking tobacco during pregnancy is related to not only increased infant mortality and decreased
birth weight [10–12] but also to adverse behavioral outcomes [13]. The children of women who smoke are more likely to develop cognitive deficits [14–17], including ADHD [18], impaired attention and orientation [11, 19–21], poor impulse control [22], hyperactivity, and learning impairment [23]. Moreover, the behavioral effects of prenatal TS exposure are long-lasting and likely permanent [24].

Some of these outcomes have been associated with changes in DNA methylation during fetal [25, 26] and adolescent [27] development. For example, low birth weight in male offspring of smoking mothers was associated with increased DNA methylation at the growth hormone-insulin-like growth factor II (IGF2) [26]. The affected region was further shown to have marked effects on IGF2 transcription, with a 1% change in methylation leading to a halving (increased methylation) or doubling (decreased methylation) of transcript levels [26]. Gestational TS exposure was also associated with increased DNA methylation of the gene encoding brain-derived neurotrophic factor (BDNF) in adolescents [27], a gene that was also found to serve as a methylation biomarker for depression [28]. Epigenome-wide alterations have also been associated with in utero tobacco smoke exposure [29, 30]. Altered DNA methylation at 26 CpGs across 10 genes in infant cord blood was associated with smoking during pregnancy [29, 30]. Affected genes included the aryl hydrocarbon receptor repressor (AHR), the cytochrome P450 family 1 subfamily A member 1 (CYPIA1), both of which play a role in the detoxification of deleterious TS constituents [30].

Maternal smoking has also been associated with altered dietary patterns such as the Mediterranean diet [31] and levels of various micronutrients that are essential for healthy fetal development [32–34]. For example, folic acid (vitamin B9) and vitamins B12 and B6 are necessary components of the one-carbon metabolism pathway that generates methyl groups used for DNA methylation and other cellular methylation reactions. Maternal serum levels of these and other micronutrients, such as vitamin D, are lower in pregnant smokers [32–34]. While substantial evidence exists supporting the interaction between maternal micronutrient levels and fetal DNA methylation patterns [35], little is known about how smoking-related alterations in micronutrient levels impact DNA methylation at MEs.

Here, we leveraged multiple human tissue sources to validate putative human MEs and examine ME DNA methylation within the context of TS exposure [5, 6]. We first used male and female conceptual tissues derived from the mesoderm, endoderm, and ectoderm to validate ME status for three previously identified MEs, these samples were excluded from the final analyses, giving a final sample size of 85. NEST study protocols were approved by the Duke University Institutional Review Board (Pro00014548). All enrolled women provided written informed consent for their and their child’s participation.

**Methods**

**Human Conceptual Tissues**

Human brain, liver, and kidney conceptual tissues (six males, six females) were obtained between the years of 1999 and 2010 from the NIH-supported Laboratory of Developmental Biology at the University of Washington and used under a protocol approved by the Duke University Institutional Review Board (Pro00014066). Tissue specimens were stored at –80°C until required for DNA and RNA extraction.

**Study Population: NEST**

Pregnant women were recruited from central North Carolina for the NEST from prenatal clinics at Duke University Hospital and Durham Regional Hospital obstetrics facilities between 2005 and 2011. All participants spoke English and/or Spanish and were at least 18 years of age. Women completed a questionnaire in English or Spanish at the time of recruitment, which assessed sociodemographic characteristics including age, race, marital status, level of education, self-reported cigarette smoking, and/or exposure to TS. There were 2640 women who participated in the study. For our current analyses, a subset of 90 participants were randomly chosen based on maternal plasma cotinine levels measured during gestation (described below) to achieve a roughly equal distribution of smokers (N = 48) and nonsmokers (N = 42). Of these 90, five had a body mass index (BMI) indicative of being underweight (<18.5). Because our small sample size in this category did not give us the ability to control for potential confounding by underweight status, these samples were excluded from the final analyses, giving a final sample size of 85. NEST study protocols were approved by the Duke University Institutional Review Board (Pro00014548). All enrolled women provided written informed consent for their and their child’s participation.

**Maternal Plasma Cotinine Measurement**

Whole blood was collected in 7 ml ethylenediaminetetraacetic acid (EDTA) containing vacutainer tubes from NEST participants during pregnancy (mean gestational age of sampling in 85 samples examined = 120 days). One milliliter of whole blood was removed from the vacutainer tube, aliquoted, and stored at −80°C for later measurement of red blood cell folate levels (see below). The remaining whole blood was centrifuged to separate the red cells from the plasma and leukocyte fractions and the latter two were aliquoted and stored at −80°C. Cotinine is a relatively stable metabolite of nicotine and serves as a blood biomarker of TS exposure [36]. Due to their hydrophobicity, both nicotine and cotinine readily cross the placenta [37] and accumulate in the fetus if exposure is sustained throughout pregnancy [38]. Cotinine (ng/ml plasma) was measured by Dr JunFeng Zhang’s Laboratory at Duke University via methods previously described [39] using high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS–MS). The limit of detection was 0.05 ng/ml, with reproducibility greater than 94%. Based on measured cotinine levels, participants were dichotomized into two exposure categories: (i) “no exposure,” or women with plasma cotinine levels below 1 ng/ml; and (ii) “active smoking”, or women with plasma cotinine levels greater than 3 ng/ml. These categorizations are congruent with previous NEST studies [39] and with studies examining passive smoke exposure during pregnancy [40, 41].

**Maternal Whole Blood Micronutrient Measurement**

Red-blood cell (RBC) folate levels were estimated from maternal whole blood samples collected at the time of enrollment into the study using a commercial kit, with concentrations accounting for hematocrit levels, as previously described [42]. Maternal plasma vitamin B12 levels were measured using a commercially available immunoassay, as previously described [43]. Plasma vitamin B6 pyridoxal 5′-phosphate (PLP) and pyridoxic acid (PA) as
well as homocysteine levels were measured using HPLC techniques as previously described [43]. Plasma 25-hydroxyvitamin D [25(OH)D] levels were also measured using a commercially available immunoassay as described [44].

**Umbilical Cord Blood**

Venous umbilical cord blood samples were collected at delivery into EDTA containing vacutainer tubes for NEST participants. Samples were separated by centrifugation into RBCs, plasma, anduffy coat as previously described [42]. Cord blood components were aliquoted and stored at −80°C until processed for DNA extraction.

**Separation of Major Umbilical Cord Blood Cell Fractions**

Polymorphonuclear cells (PMNs) and peripheral blood mononuclear cells (PBMCs) were fractionated from newborn umbilical cord blood as previously described [45]. In brief, specimens were fractionated using Lympholyte®-poly (Cedarlane Laboratories Limited, Burlington, NC, USA) to separate and collect the PMN and PBMC fractions. Fraction purity was assessed following Giemsa staining.

**DNA/RNA Isolation and Bisulfite Modification**

Both genomic DNA and total RNA were extracted from conceptual tissue samples using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen). Genomic DNA was extracted from theuffy coat of NEST infant cord blood and PMN/PBMC fractions using the Qiagen QIAamp DNA Mini kit (Qiagen; Valencia, CA). DNA and RNA samples were eluted in nuclease-free water and nucleic acid concentration and purity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA). Genomic DNA (800 ng) was modified by bisulfite treatment for subsequent DNA methylation analysis using the Zymo EZ DNA Methylation Kit (Zymo Research, Inc.; Irvine, CA) and eluted in 40 µl nuclease-free water for a final concentration of 20 ng/µl bisulfite-treated DNA, assuming complete recovery.

**DNA Methylation Analyses**

Bisulfite pyrosequencing was used to analyze CpG methylation as described [26] for the following MEs: BOLA3, PAX8, SLITRK1, and ZFYVE28 using primers and polymerase chain reaction (PCR) protocols of previously published assays [6]. Primers were ordered from Sigma Aldrich (Saint Louis, MO, Supplementary Table S1). All pyrosequencing assays were performed using a Qiagen PyroMark Q96 MD Pyrosequencer and validated for quantitative performance using methylation standards (Qiagen EpiTect Control DNA Set, Human Cat No. S9568) of defined proportions (0%, 25%, 50%, 75%, and 100% methylated). These defined standards were used to validate the linearity and range of the ME assays (Supplementary Fig. S1). Average methylation was calculated across all CpGs within each ME region analyzed using PyroQ CpG Software (Qiagen) and the averages were used as the unit of measurement, consistent with prior reports [6]. Each pyrosequencing assay contained 7 µl PCR product generated using the following conditions for BOLA3, PAX8, SLITRK1, and ZFYVE28: 95°C for 15 min, then 55 cycles of 94°C for 30 s, (assay-specific) annealing temperature for 30 s, 72°C for 30 s, followed by 72°C for 10 min. Annealing temperatures were as follows: BOLA3, 64°C; PAX8, 67°C; SLITRK1, 61°C; ZFYVE28, 59°C (Supplementary Table S1).

**Gene Expression Analyses**

PAX8 transcript levels were measured in conceptual brain, liver, and kidney tissues using TaqMan Reverse-Transcription Real-Time PCR using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). One-step cDNA synthesis was performed using the qScript XLT One-Step RT-qPCR Kit from Quanta BioSciences (Beverly, MA) and the following thermal cycler conditions: 50°C for 10 min, 95°C for 1 min. Real-time PCR cycling immediately followed using conditions: 40 cycles of 95°C for 10 s, then 60°C for 60 s. TaqMan assays included: PAX8: Hs00247586_m1, B2M: Hs00187842_m1 (Thermo Fisher Scientific). PAX8 transcript levels were calculated as the difference in cycle threshold (Ct) values relative to beta-2 microglobulin (B2M). PAX8 transcript levels were correlated to DNA methylation levels for each sample using Spearman’s correlation.

**Statistics**

Preliminary analyses examining the distributions of all outcomes indicated that many outcomes were not normally distributed, so we used data transformations and nonparametric statistical models to assess associations where appropriate. We first examined associations between sociodemographic variables and maternal nutrient levels, smoking status, and ME methylation using Spearman’s correlation analyses, linear regression models, and chi-squared tests. Maternal sociodemographic variables examined included race, education, preconceptional BMI, and age and were categorized as listed in Table 1. We examined the effects of TS exposure (categorized as exposed and unexposed) and micronutrients (categorized into tertiles, representing low, mid, and high intake) on individual ME methylation status using generalized linear models, accounting for potential confounding by sociodemographic characteristics and maternal nutrition. We next looked at methylation across all MEs using a mixed modeling approach [6]. In this model, ME name and individual were run as random effects. Maternal sociodemographic factors were considered as possible confounding variables in regression analyses, although none of the confounders under consideration were associated with both exposure (TS) and ME methylation in bivariate analyses. We conducted multivariate analyses using a backward covariate selection approach and retained covariates that were significantly associated with ME methylation in adjusted models. All statistical analyses were performed using JMP Pro Version 13.0.

**Results**

**Validation of MEs at BOLA3, PAX8, and ZFYVE28**

Upon successful validation of ME pyrosequencing assays [6] (Supplementary Fig. S1, Supplementary Table S1), we confirmed ME status for BOLA3, PAX8, and ZFYVE28 in human conceptual brain, liver, and kidney tissues (Fig. 1). We found significant within-individual intertissue correlation (P < 0.02) for all three MEs examined except at ZFYVE28 in brain tissue. However, due to the high correlation between ZFYVE28 CpG methylation in liver and kidney tissues, strong interindividual variation and congruence with previous determinations for comprising an ME [6], our data support that ZFYVE28 comprises an ME. We also examined SLITRK1, another putative human ME [6], and were able to demonstrate strong intertissue correlation but rather minimal interindividual variation (range ~3%, Supplementary Fig S2a and b). While methylation at SLITRK1 was highly correlated in liver versus brain comparisons, correlations were weaker for kidney versus liver and kidney versus brain, with one outlying data point driving the
associations. Further, examination of SLITRK1 ME methylation in cord blood showed very low overall levels of methylation with little interindividual variation (e.g. see Supplementary Fig. S2C), altogether inconsistent with ME status for SLITRK1.

**NEST Maternal Demographics and Smoking Status**

The maternal sociodemographic breakdown of NEST women in our study sample is outlined in Table 1, and additional gestational characteristics are described in Supplementary Table S2. Samples examined were derived from predominantly black (56%) or white (41%) mothers with a wide distribution of education levels, preconceptional BMIs, and ages (Table 1). Offspring were 40% male and 60% female (Supplementary Table S2). Maternal plasma was collected during pregnancy at a mean gestational age of 119.6±8.7 days [standard error of the mean (SEM); Supplementary Table S2]. Mean plasma cotinine across all women was 55.9±8.5 ng/ml (SEM). Among women defined as “unexposed” to TS (N = 38), gestational mean plasma cotinine was 0.17±0.04 ng/ml, and for those “exposed,” (N = 47), 100.9±11.7 ng/ml. The distribution of cotinine values within the subset of samples examined (N = 85) was highly correlated to that of the samples within the entire cohort for which cotinine values were measured (N = 855) (R_s = 0.36, P = 0.0007) (Supplementary Table S3).

Maternal education, commonly used as a proxy for socioeconomic status (SES), was strongly associated with maternal smoking status (P<0.0001, two-sided chi-square test, Table 1). Maternal smoking status was also significantly associated with lower infant birth weight (P = 0.0002, Supplementary Table S4), consistent with previous findings [26].

**Maternal Micronutrient Levels, Smoking Status, and Sociodemographic Variables**

Micronutrient measurements were available for 41 to 44 of the N = 85 samples examined. Mean plasma micronutrient levels were as follows: RBC folate, 218.6±11.9 µg/l; vitamin B6 PLP 9.6±1.2 nmol/l; vitamin B6 PA, 6.0±1.1 nmol/l; vitamin B12, 524.1±36.9 ng/ml; 25(OH)D, 16.13±0.9 ng/ml; and homocysteine, 0.8±0.03 µg/ml. Smoking exposure was significantly associated with reduced levels of RBC folate (P = 0.02), plasma vitamin B6 PLP (P = 0.05), and plasma vitamin B12 (P = 0.007, Table 2), as expected based on previous studies [32, 34]. Correspondingly, smoking exposure was also associated with increased plasma homocysteine levels (P = 0.02, Table 2), a common indicator of low B vitamin levels, including folate [46].

The micronutrients folate and vitamins B6 and B12 are critical for the generation of methyl groups for DNA and other methylation reactions and are essential for normal embryonic and fetal development [35, 43, 46, 47]. All of these micronutrients are recommended as dietary supplements before and during pregnancy [47]. The Institute of Medicine (IOM) has defined RBC folate deficiency as a level less than 140 µg/l, and plasma or serum vitamin B12 deficiency as levels less than 200 ng/ml [48]. Vitamin B6 PLP, the most commonly measured vitamin B6 derivative and indicator of B6 status, is considered deficient in the plasma at levels less than 20 nmol/l [47], and vitamin D 25(OH)D is considered deficient in the plasma at levels less than 20 ng/ml [49]. Using these deficiency thresholds, we found 2.3% of pregnant women examined to be vitamin B12 deficient, 14.0% to be folate deficient, 76.7% to be vitamin D deficient, and 90.9% to be PLP deficient. Smoking exposure was significantly associated with PLP deficiency (P = 0.015, two-sided chi-square test of micronutrient deficiency status vs. smoking exposure status) and less so with RBC folate or vitamin D deficiencies (P = 0.06 and P = 0.12, respectively).

Upon examination of relationships between sociodemographic covariates, education (SES) was most strongly associated with micronutrient levels (Supplementary Table S5). Mothers who received less than a college education had significantly lower total folate (P = 0.03), vitamin B6 PLP (P = 0.0002), and vitamin B6 PA (P = 0.003) compared to those with at least some college education, as well as higher homocysteine (P = 0.01), an indicator of reduced vitamin B levels (Supplementary Table S5). Mothers with a pre-pregnancy BMI indicative of overweight or obesity (>25) had significantly less plasma vitamin B6 PLP (P = 0.04) and vitamin B12 (P = 0.003, Supplementary Table S6) than those with a normal BMI (18.5–25). Further, mothers who identified as nonwhite had significantly lower vitamin D levels (P = 0.0036) than those who identified as white (Supplementary Table S7). Racial disparities in maternal vitamin D levels have previously

### Table 1: NEST cohort maternal sociodemographic data stratified by plasma cotinine levels

| Race | Education | BMI | Age | Full sample | Unexposed | Exposed | \( P \) |
|------|-----------|-----|-----|-------------|------------|----------|------|
| White | No college | 18.5 < x < 25 (normal) | < 25 y.o. | 34 (40.00) | 19 (50.00) | 15 (31.92) | 0.1199 |
| Black | At least some college | > 25 (overweight) | 25–30 y.o. | 48 (56.47) | 17 (44.74) | 31 (65.96) | 0.0002 |
| Hispanic | More than college | > 25 (overweight) | > 30 y.o. | 2 (3.5) | 1 (2.63) | 1 (2.13) | <0.0001 |
| Other | Age <25 y.o. | | | 1 (1.18) | 1 (2.63) | 0 | - |

**Notes:**
- Unexposed: maternal plasma cotinine <1 ng/ml.
- Exposed: maternal plasma cotinine >3 ng/ml.
- Nontransformed cotinine values presented.
- \( P \)-value for two-sided chi-square test of maternal sociodemographic variable (race, education, BMI, age) vs smoke exposure status (exposed or unexposed).
Figure 1: Intertissue correlation and interindividual variation verify BOLA3, PAX8, and ZFYVE28 as human MEs. (A) Scatter plots depict interindividual variation and intertissue correlation in DNA methylation (represented as average % methylation) at PAX8, BOLA3, and ZFYVE28 in brain, liver, and kidney tissue, derived from the three embryonic germ layers. N = 6 male, N = 6 female. (B) Pairwise comparisons across conceptual tissues reveal strong intertissue correlation at MEs.

Maternal TS Exposure is Associated with Increased Infant ME DNA Methylation

To determine if differences exist in DNA methylation at the MEs in the major leucocyte blood cell fractions found in umbilical cord blood, [53], we evaluated variability in CpG methylation at BOLA3, PAX8, and ZFYVE28 in PMNs (basophils, neutrophils, and eosinophils) and PBMCs (monocytes and lymphocytes) using the validated ME pyrosequencing assays. CpG methylation was highly correlated across cord blood PMN and PBMC fractions for all three MEs (BOLA3 $R_S = 0.91$, $P < 0.001$; PAX8, $R_S = 0.93$, $P < 0.001$; and ZFYVE28, $R_S = 0.97$, $P < 0.001$; Fig. 2A and B). These strong correlations not only further support the ME status of BOLA3, PAX8, and ZFYVE28 but also justify the examination of ME DNA methylation in mixed leucocyte populations in unfractionated umbilical cord blood.

To determine if there is a relationship between maternal smoking and infant ME DNA methylation, we measured ME methylation in DNA isolated from NEST infant cord blood buffy coat, which contains a mixture of leucocyte types. DNA methylation was significantly increased in the exposed compared to unexposed group for the PAX8 ME, after adjusting for maternal age.

| Metastable Epialleles | Kidney vs. Liver | Liver vs. Brain | Kidney vs. Brain |
|-----------------------|-----------------|----------------|-----------------|
|                       | $R_S$           | $P$            | $R_S$           | $P$            | $R_S$           | $P$            |
| BOLA3                 | 0.8811          | 0.0002         | 0.8601          | 0.0003         | 0.8881          | 0.0001         |
| PAX8                  | 0.9650          | <0.0001        | 0.8601          | 0.0003         | 0.8881          | 0.0001         |
| ZFYVE28               | 0.6643          | 0.0185         | 0.5175          | 0.0849         | 0.2448          | 0.4433         |
and maternal plasma vitamin D levels, PAX8 average DNA methylation was 5.22% higher in the exposed compared to unexposed group (95% CI: 0.33% to 10.10%; \( P = 0.037 \); Fig. 2D and E). Average DNA methylation at BOLA3 and ZFYVE28 did not differ significantly between exposed and unexposed groups, although DNA methylation across each ME was higher in exposed compared to unexposed groups (BOLA3: 0.72% higher in exposed vs. unexposed; 95% CI: –1.95% to 3.40%; \( P = 0.59 \); ZFYVE28: 1.05% higher in exposed vs. unexposed; 95% CI: –0.67% to 2.77%; \( P = 0.23 \); confounders were not identified for DNA methylation status at BOLA3 or ZFYVE28 in bivariate or backward elimination regression analysis). After adjusting for maternal plasma vitamin B6 PA levels, we found a significant overall effect of smoking exposure on DNA methylation in a combined analysis of all three MEs using a repeated measures mixed regression model, as was done in the original report defining these regions as MEs [6]. Exposure was related to an average 2.38% increase in methylation across all three MEs (95% CI: 0.14% to 4.64% methylation; \( P = 0.038 \); Fig. 2D and E).

**ME DNA Methylation and Maternal Micronutrient Levels**

Due to the divergent associations observed between maternal smoking status versus plasma micronutrient levels and ME DNA methylation, we investigated potential relationships between maternal smoking, micronutrient levels, and cord blood ME DNA methylation in the 85 NEST samples examined. We observed a trending negative association between maternal micronutrient levels and ME DNA methylation at PAX8 for folate (PAX8% ME methylation versus total folate: \( R_S = -0.25 \), \( P = 0.10 \)) and a significant negative association at BOLA3 for vitamin D (BOLA3% ME methylation versus vitamin D: \( R_S = -0.36 \), \( P = 0.02 \); Supplementary Table S9). However, after adjusting for maternal TS exposure using a mixed linear regression model, there were no statistically significant associations between micronutrients and ME methylation (data not shown). Our findings indicate that maternal smoking is driving the association between maternal micronutrient levels and DNA methylation at MEs and that maternal micronutrient levels in this case do not independently influence DNA methylation levels at MEs. Further, the observed negative trends between ME DNA methylation and maternal micronutrient levels before adjusting for maternal smoking suggests that smoking-induced increases in DNA methylation at MEs can be established despite methyl-donor micronutrient deficiencies.

**PAX8 mRNA Levels are Negatively Associated with DNA Methylation**

Due to the significant effect of smoking exposure on DNA methylation at PAX8, we sought to determine if there was a potential functional relationship between PAX8 ME DNA methylation and PAX8 gene expression. Using reverse-transcription real-time PCR of total RNA derived from human conceptal brain, liver, and kidney tissues and normalized for RNA input, we found significantly higher levels of PAX8 mRNA transcripts in the kidney relative to liver tissue (\( P < 0.0001 \), Fig. 3A). Due to the increased expression in kidney, we next evaluated a potential functional relationship between PAX8 mRNA content and ME DNA methylation in conceptal kidney tissues. Normalized PAX8 mRNA levels were negatively associated with PAX8 ME DNA methylation in kidney; however, this association was not statistically significant (\( R_S = 0.5545 \), \( P = 0.0767 \); Fig. 3B). Using the slope of the correlation curve (\( y = 21.43x + 4.988 \)) and assuming complete doubling in each PCR cycle, we found that, on average, a 21.4% change in PAX8 ME methylation in the kidney corresponded to a halving or doubling of the PAX8 mRNA content. These data support that the normal distribution of PAX8 ME methylation is associated with a four-fold range of PAX8 expression and that the random establishment of methylation at this locus naturally produces wide, inherent variation between individuals in the expression of this functionally important gene.

**Discussion**

The periconceptional establishment of DNA methylation at human MEs can be vulnerable to environmental factors. Because DNA methylation at MEs is highly correlated across all tissue types, perturbations to their establishment can result in somatic effects. While environmental influences, like the periconceptional availability of nutrients, have been shown to alter DNA methylation at MEs [5, 6], little is known about their vulnerability to in utero exposures like TS. Our data indicates that nearly 21% of women of childbearing age smoke while pregnant in central North Carolina, and around 15% of pregnant women are exposed to second-hand smoke (Supplementary Table S3) [39]. Compared to nation-wide estimates based on self-report, smoking during pregnancy is nearly three times more common in our study sample [54]. Given the prevalence of maternal smoking (Supplementary Table S3) as well as known associations between developmental TS exposure and alterations in cord blood DNA methylation [25–27, 30, 54], we examined relationships

### Table 2: NEST cohort maternal micronutrient levels stratified by TS exposure

| Nutrient | Unexposed\( ^a \) | Exposed\( ^b \) |
|----------|-------------------|------------------|
|          | N total | N (%) | Nutrient mean ±SEM | N (%) | Nutrient mean ±SEM | \( P \) |
| Total folate (µg/l) | 43 | 22 (51.16) | 246.38 ± 13.09 | 21 (48.84) | 189.41 ± 18.33 | 0.0147 |
| B6 PLP (nmol/l)\( ^d \), \( ^e \) | 44 | 22 (50.00) | 13.27 ± 2.00 | 22 (50.00) | 5.93 ± 1.01 | 0.0497 |
| B6 PA (nmol/l)\( ^d \), \( ^e \) | 44 | 22 (50.00) | 7.71 ± 1.54 | 22 (50.00) | 4.35 ± 1.50 | 0.4434 |
| Vitamin D (ng/ml)\( ^f \) | 43 | 21 (48.84) | 17.62 ± 1.32 | 22 (51.16) | 14.70 ± 1.16 | 0.3087 |
| Vitamin B12 (ng/l) | 44 | 22 (50.00) | 620.41 ±54.94 | 22 (50.00) | 427.77 ± 40.83 | 0.0074 |
| Homocysteine (µg/ml)\( ^d \), \( ^e \) | 41 | 20 (48.78) | 0.70 ± 0.04 | 21 (51.22) | 0.79 ± 0.05 | 0.0193 |

\( ^a \)Unexposed: maternal plasma cotinine <1 ng/ml.
\( ^b \)Exposed: maternal plasma cotinine >3 ng/ml.
\( ^c \)Nontransformed micronutrient values presented.
\( ^d \)B6 PLP, B6 PA, and homocysteine values were natural log transformed after adding a constant prior to statistical testing to increase normality.
\( ^e \)B6 PLP, B6 PA, and homocysteine associations adjusted for maternal education.
\( ^f \)Vitamin D association adjusted for maternal race.
\( ^p \)Value for comparison between the maternal micronutrient level and smoke exposure status.
Altered metastable epiallele methylation with maternal smoking

A.

![Graphs showing average % methylation in PBMCs for BOLA3, PAX8, ZFYVE28](image)

**Average % Methylation PBMCs**

B.

| Metastable Epiallele | $R_s$ | $P$   |
|----------------------|-------|-------|
| BOLA3                | 0.91  | <0.0001 |
| PAX8                 | 0.93  | <0.0001 |
| ZFYVE28              | 0.97  | <0.0001 |

$R_s$ = Spearman's Correlation, $P$ = p value, N=14 individuals (fetal conceptual)

C.

![Timeline showing conception, weeks of gestation, ME methylation establishment, maternal plasma collection, and birth](image)

D.

![Graphs showing average % methylation by exposure status for BOLA3, PAX8, ZFYVE28](image)

**Figure 2:** (Continued)
Exposure vs. Unexposed

| Metastable Epialleles | *P   | Avg. % Diff | 95% C.I. | Covariate Adjustments |
|----------------------|------|-------------|----------|-----------------------|
| BOLA3                | 0.59 | 0.72 %      | -1.95 to 3.40 % | None |
| PAX8                 | 0.037| 5.22 %      | 0.33 to 10.10 % | Maternal Age, Vitamin D |
| ZFYVE28              | 0.23 | 1.05 %      | -0.67 to 2.77 % | None |
| All Three            | 0.038| 2.38 %      | 0.14 to 4.64 % | Vitamin B6 Pyridoxic Acid |

Figure 2: NEST maternal TS exposure is associated with increased infant cord blood DNA methylation at MEs. (A–B) Correlations in DNA methylation at BOLA3, PAX8, and ZFYVE28 across polymorphonuclear and mononuclear cell fractions (PMNs and PBMCs), supporting whole buffy coat sampling methods (*P < 0.0001, Spearman’s correlations). (C) Timeline for NEST maternal plasma and cord blood sample collection in the context of ME establishment. (D–E) Average % DNA methylation at ME loci in NEST infant cord blood stratified by maternal TS exposure status. “Unexposed” samples identified by maternal plasma cotinine levels <1 ng/ml. “Exposed” samples identified by maternal plasma cotinine levels >3 ng/ml. BOLA3: N = 36 unexposed, 40 exposed; DNA methylation 0.72% higher in exposed vs. unexposed, 95% CI: −1.95% to 3.40%; *P = 0.59. PAX8: N = 38 unexposed, N = 47 exposed; DNA methylation 5.22% higher in exposed vs. unexposed, 95% CI: 0.33% to 10.10%; *P = 0.037. ZFYVE28: N = 36 unexposed, 39 exposed; DNA methylation 1.05% higher in exposed vs. unexposed, 95% CI: −0.67% to 2.77%; *P = 0.23. All Three: N = 110 unexposed, 126 exposed; DNA methylation 2.4% higher in exposed vs. unexposed, 95% CI: 0.14% to 4.64%; *P = 0.038. Error bars represent the SEM. PAX8 association adjusted for maternal age and plasma vitamin D levels. Repeated measures association across all three MEs adjusted for maternal vitamin B6 PA levels. *P-value for comparison between infant average ME % methylation and maternal smoke exposure status using linear regression models (PAX8, BOLA3, ZFYVE28) or mixed models with repeated measures (all three MEs).

between developmental TS exposure and DNA methylation at human MEs. Because maternal smoking is also associated with decreased micronutrient levels [32, 34], particularly those that function in the generation of methyl group donors, we further examined the potential effects of smoking-related maternal micronutrient reductions on DNA methylation at infant MEs. This is the first study to demonstrate the vulnerability of DNA methylation establishment at human MEs to developmental TS exposure and with respect to TS exposure-related maternal micronutrient reductions.

Here, we leveraged multiple human tissue sources to validate three previously identified putative human MEs (BOLA3, PAX8, and ZFYVE28) and examined ME DNA methylation within the context of TS exposure [5, 6]. Analysis of human conceptual tissues allowed for examination of ME DNA methylation in tissues derived from the mesoderm, endoderm and ectoderm without the potential confounds of ex utero exposures. We observed similar ranges of ME DNA methylation in these tissues as that reported in previous adult human ME studies [5, 6], corroborating the notion that the early establishment of DNA methylation at MEs leads to methylation levels that are stable throughout germ layer specification, tissue differentiation, and during subsequent somatic cell division throughout the life course. These tissues additionally enabled us to examine the tissue-specific, functional roles of ME DNA methylation during fetal development.

The NEST cohort samples enabled us to examine infant ME methylation in the context of matched maternal micronutrient levels, as well as sociodemographic variables like race, maternal education level, and pre-pregnancy BMI. From these data sets, we examined potential confounding effects of sociodemographic variables and micronutrient levels on infant cord blood ME DNA methylation. For example, offspring methylation at PAX8 was significantly associated with maternal age and vitamin D levels, underscoring the importance of collectively examining these covariates in human DNA methylation studies (Fig. 2D and E).

Among the sociodemographic variables analyzed, education level, an indicator of SES, was most strongly associated with maternal tobacco smoke exposure status as has been previously shown [26, 39] (Table 1). Upon cross-examination of sociodemographic variables and maternal micronutrient levels independent of exposure status, education level was again most strongly associated with decreased micronutrient levels, significantly impacting three of the five micronutrient levels examined. Correspondingly, education level was significantly associated with increased homocysteine levels (Supplementary Table S5). Within our study, these findings position SES as the most impactful sociodemographic risk factor with regard to smoking and maternal micronutrient levels.

After adjusting for potential confounding by sociodemographic factors, maternal TS exposure status was significantly correlated with reduced gestational levels of RBC folate (consistent with previous findings [39]), vitamin B6 PLP, and vitamin B12, as well as increased levels of homocysteine, an indicator of low vitamin B and total folate levels (Table 2). All of these micronutrients derived from dietary and supplementation sources are involved in one-carbon metabolism, a metabolic pathway that generates substrates used for methylation reactions in the cell, including DNA methylation [46]. Methyl group-generating micronutrients are essential for normal development [35, 43, 46, 47] and the prevention of neurodevelopmental abnormalities like neural tube defects. As such, women of childbearing age are strongly recommended to consume a diet with sufficient levels of vitamins B6, B12, and folate when planning pregnancy and throughout gestation. Despite these efforts, many women of childbearing age do not receive adequate levels of methyl donor micronutrients through diet alone [47]. Therefore, in 1998 the US Food and Drug Administration required the fortification of enriched grain products with folic acid to help ensure adequate levels in the event a woman becomes pregnant. This requirement was put in effect in part because nearly half of all pregnancies, at least in the USA,
Based on the IOM’s defined thresholds for micronutrient sufficiency, more than three quarters of women examined in our study were deficient for both vitamin D and PLP. Vitamin D deficiencies during pregnancy have been associated with miscarriage, preeclampsia, and stunted fetal growth [44, 51, 52, 55]. Although PLP deficiency is considered rare due to the nearly ubiquitous presence of B6-rich foods, deficiency is higher among obese and pregnant individuals [56]. As 61.7% of the pregnant women in our study were obese, the widespread PLP deficiency (90.9%) we observed is perhaps unsurprising. In line with previous findings [57], PLP deficiency was significantly associated with smoking status ($P = 0.01$), with lower plasma PLP levels in mothers exposed to TS. Vitamin B6 is critical to neurotransmitter synthesis and normal fetal neurodevelopment. As such, vitamin B6 deficiency has been associated with the development of neurological disorders, such as seizure disorder [56]. However, adverse clinical outcomes due to PLP deficiency in infants remain contested and poorly characterized [47].

We are the first to identify a significant association between maternal smoking and elevated DNA methylation at the PAX8 ME ($P = 0.037$, Fig. 2D and E) and across all three MEs collectively ($P = 0.038$, Fig. 2D and E) in infant cord blood. The observed additive effect on DNA methylation across multiple ME regions suggests their enhanced vulnerability to the early life in utero environment and maternal exposures. These findings are consistent with previous reports of enhanced exposure effects across multiple ME regions [5, 6] and together support the idea that there is general vulnerability of MEs to environmental exposures rather than specific ME targets. As all associations were corrected for the potential confounding effects of maternal micronutrient levels, they stand independent of smoking-related decreases in maternal micronutrient levels.

Increasing methylation at both PAX8 and BOLA3 were associated with decreasing maternal levels of total folate and vitamin D, respectively. While higher maternal micronutrient levels, especially those involved in one-carbon metabolism, have been associated with increased infant DNA methylation at imprinted loci [43, 57], this is not always the case [5, 6, 35, 42, 46, 58, 59]. Maternal folate levels were negatively associated with fetal DNA methylation at several imprinted loci in the NEST cohort [42, 58] and at putative human MEs [5, 6]. Our findings corroborate these studies and indicate that increased DNA methylation can occur under developmental conditions of maternal methyl donor deprivation. Such paradoxical findings have also been reported in cancer models and suggest that the effects of folate levels on DNA methylation are site-specific rather than epigenome-wide [60, 61]. While developmental vitamin D depletion has been generally associated with hypomethylation [62, 63], these associations were locus-specific [44]. Our findings are the first to demonstrate an inverse relationship between developmental vitamin D levels and DNA methylation at MEs. The inverse relationships we observed between DNA methylation at MEs and micronutrient availability versus maternal TS exposure merits further study into whether and how different exposures alter DNA methylation at MEs in the early embryo, especially within the context of methyl donor deprivation.

Since methylation at PAX8 indicated the strongest independent association to exposure ($P = 0.038$, Fig. 2D and E), we further explored its functional role in conceptual tissues derived from the three germ layers (mesoderm-kidney, ectoderm-brain, endoderm-liver). Additionally, due to the proximity between PAX8’s ME region, transcriptional start site, and large, intragenic CpG island [9], we hypothesized that DNA methylation at the PAX8 ME may

**Figure 3:** PAX8 mRNA levels and association with ME DNA methylation in conceptual kidney tissue. PAX8 mRNA levels were calculated as the difference in the cycle threshold (Delta Ct) between PAX8 and B2M following reverse-transcription real-time PCR. (A) PAX8 mRNA levels differ significantly across tissue types, with the highest mRNA content in the kidneys ($**P \leq 0.0001$, two-way ANOVA followed by Tukey’s HSD post-hoc test). Error bars represent the SEM. $N = 6$ male, $N = 6$ female. (B) Correlation of PAX8 ME average % DNA methylation and Delta Ct in kidney conceptual tissue. Correlations assessed using Spearman’s Correlation ($R_s$). $N = 6$ male, $N = 5$ female.

are unplanned [47]. In addition, the post-fertilization period of epigenetic reprogramming likely also requires an adequate supply of methyl group donor micronutrients to support the re-methylation of the peri-implantation embryo. This reprogramming occurs well before most women suspect they may be pregnant. Hemodilution of micronutrients during pregnancy-related blood volume gain exacerbates this deficit and could impact the ability to maintain methylation profiles established earlier in development in rapidly dividing cells during organ growth.
functionally contribute to regulation of gene expression. PAX8 is part of the paired-box transcription factor family, a group of genes involved in organogenesis during development [64, 65], and has specifically been associated with thyroid, brain, and kidney development [64, 65]. As such, PAX8 has also been implicated in promoting tumor cell growth in adults [64, 66], and its expression has been linked to thyroid cancer [64], kidney cancer [64, 65, 67], and ovarian cancer [64, 66, 68]. PAX8’s established role in a number of cancers provided additional impetus to investigate a potential relationship between PAX8 ME methylation and gene expression within the context of developmental TS exposure, especially since TS exposure itself is an established cancer risk factor.

Although PAX8 ME methylation was highly correlated across tissues (Fig. 1), we resolved a tissue-specific difference in PAX8 gene expression, which may be important depending on gene function and the interindividual distributional range of ME DNA methylation (Figs 1 and 3). PAX8 transcript levels were highest in the kidneys (P < 0.0001) and lowest in the liver [69] (Fig. 3A). These results were unsurprising given the developmental role of PAX8 in kidney formation [64, 65] and in cancer of the kidneys [64, 65, 67]. PAX8 transcript levels were negatively associated with ME DNA methylation in conceptal kidney tissues. Normalized PAX8 cycle threshold (Ct) values indicated that every 21.4% difference in PAX8 methylation corresponds to a two-fold difference in PAX8 expression. Our results indicate that the normal range of PAX8 methylation spans from ~20% to ~90%. This range suggests that there is normally high variability in PAX8 expression. This is relevant given the role of PAX8 in cancer [64–68]. It would be of interest to determine if individuals with lower baseline levels of methylation at the PAX8 ME locus are at increased risk of cancer or have a more aggressive course of disease.

Our study was primarily limited by low sample size for some analyses, especially after adjusting for covariates. Due to limited sample availability, we were also under-powered in our gene expression analysis in conceptal tissues, which likely accounts for the inability to resolve statistical significance. Further work is needed to better understand how ME DNA methylation may functionally contribute to gene expression, perhaps through mediating transcription factor binding. In addition, while we were able to observe changes in maternal micronutrient levels associated with one-carbon metabolism, our study was limited by the fact that we could not examine potential confounding by maternal dietary supplement intake, which could have influenced our results.

Our study had several notable strengths. First, we employed quantitative sequencing methodology to validate and assess CpG methylation at putative human MEs across multiple human tissue sets, including human conceptal tissues and umbilical cord blood. Examining ME methylation in human conceptal tissues provided a unique insight into human in utero ME methylation levels, about which little is known. The fact that we observed similar ranges of ME methylation in conceptal tissue as has been previously observed in human adult tissue [5, 6] further corroborates the notion that ME methylation is stable throughout life. Access to infant cord blood samples with corresponding maternal sociodemographic information and micronutrient levels enabled an integrative analysis of exposure-related associations between micronutrient status and infant ME DNA methylation—an assessment that has not been previously performed. We were further able to cross-examine maternal sociodemographic variables with micronutrient levels to gain a better understanding of exposure-independent factors that influenced the in utero environment during the time of sampling.

In conclusion, our research revealed the potential for developmental TS exposure to alter DNA methylation in the developing fetus at vulnerable regions of the epigenome. Our findings not only support BOLA3, PAX8, and ZFYVE28 as human MEs but further indicate their individual and collective vulnerability to TS exposure during a critical early developmental window. Our integrative analyses of matched maternal sociodemographic information and micronutrient levels revealed exposure-related and -independent interactions with infant DNA methylation at human MEs, further underscoring the importance of considering these factors in epigenetic studies. While we identified a trending functional relationship between PAX8 ME DNA methylation and gene expression, more work is needed to better understand how TS exposure mechanistically contributes to increased methylation—at least for the MEs we examined, and how these changes might potentially alter risk of disease and/or its course. Further, a better understanding on the potential interaction between pre-and peri-conceptional maternal micronutrient availability and environmental exposures is paramount given the micronutrient-independent effects we observed from maternal smoking on infant ME methylation. We believe that these gaps are critical to address to better understand this potential new route to developmental origins of disease.

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Supplementary data

Supplementary data is available at EnuEpig online.

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Conflict of interest statement

None declared.

Author contributions

S.K.M. conceived the study. R.J. and S.K.M. designed the study. R.J. and C.G. performed experiments and acquired the data. R.J., K.H., and S.K.M. analyzed the data. C.H., K.H., and S.K.M. contributed reagents/materials, analysis tools. R.J. wrote the paper. All authors edited and approved the manuscript.
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