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Prenatal trimester-specific intake of micronutrients: global DNA methylation and hydroxymethylation at birth and persistence in childhood

Original Article

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Abstract

The prenatal environment may program health and disease susceptibility via epigenetic mechanisms. We evaluated associations of maternal trimester-specific intake of micronutrients with global DNA methylation (%5mC) and 5-hydroxymethylation (%5hmC) at birth in cord blood and tested for persistence into childhood. We quantified global %5mC and %5hmC in cord blood cells ($n = 434$) and in leukocytes collected in early ($n = 108$) and mid-childhood ($n = 390$) from children in Project Viva, a pre-birth cohort from Boston, MA. Validated food frequency questionnaires estimated maternal first- and second-trimester intakes of vitamin B_2, vitamin B_6, vitamin B_{12}, folate, betaine, choline, methionine, iron, and zinc. Mean (SD) cord blood %5mC and %5hmC was 5.62% (2.04) and 0.25% (0.15), respectively. Each μg increase in first-trimester B_{12} intake was associated with 0.002 lower %5hmC in cord blood (95% CI: −0.005, −0.0003), and this association persisted in early childhood ($\beta = -0.007$; 95% CI: −0.01, −0.001) but not mid-childhood. Second-trimester iron (mg) was associated with 0.01 lower %5mC (95% CI: −0.02, −0.002) and 0.001 lower %5hmC (95% CI: −0.01, −0.00001) in cord blood only. Increased second-trimester zinc (mg) intake was associated with 0.003 greater %5hmC in early childhood ($\beta = 0.003$; 95% CI: 0.0004, 0.006). Second-trimester folate was positively associated with %5hmC in early childhood only ($\beta = 0.08$, 95% CI: 0.003, 0.16). Associations did not survive multiple testing adjustment; future replication is needed. Trimester-specific nutrients may impact various sensitive windows of epigenetic programming some with lasting effects in childhood. Further research is needed to understand the role of gene-specific epigenetic changes and how global DNA methylation measures relate to child health.

Introduction

Differences in global DNA methylation in infants have been shown to be associated with maternal and child health outcomes, such as preeclampsia[1] and low birth weight.[2] Maternal nutrition and other fetal environmental factors can play critical roles in fetal development, birth weight, and long-term disease risk by perturbing epigenetic modifications during sensitive windows of fetal development.[3,4] Epigenetic programming begins in the first week of embryogenesis immediately following implantation.[5] During this process, the paternal genome experiences a rapid loss in 5-methylcytosine (5mC), whereas the maternal genome undergoes passive demethylation.[5] DNA methylation levels raise sharply during the blastocyst stage of embryogenesis, during the second week of pregnancy and continue to increase more slowly during later stages of fetal development.[5] By the blastocyte stage of development, *de novo* DNA methylation patterns are reestablished, concluding a potential sensitive window in epigenetic imprinting in early pregnancy.[5]

Diet affects availability of methyl donors and cofactors which in turn can influence epigenetic programming.[4] Therefore, epigenetic modifications might act as mediators or biomarkers between diet and various health outcomes.[4] Epigenetic modifications can occur through the

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addition of methyl groups or a hydroxymethyl group onto DNA base pairs, predominantly at Cytosines followed by a Guanine or, CpG sites. Methyl groups are acquired from methyl donors via diet; some common dietary methyl donors include folate, betaine, choline, and methionine. Methyl groups are added to the cytosine within DNA through a biological pathway known as one-carbon metabolism (OCM). Methyl groups are transferred from S-adenosylhomocysteine (SAM) to cytosine nucleotides by DNA methyltransferase (DNMT) enzymes.[4] SAM is generated by OCM, which requires methyl donors, such as methionine, folate, and choline, as well as cofactors, including zinc and vitamins B_2, B_6, and B_{12}.[4] A methylated CpG can be further modified through the addition of a hydroxyl group to create 5-hydroxymethylcytosine (5hmC). Other micronutrients also play a key role in this process. For example, Ten-Eleven Translocation (TET) enzymes, which are dependent on iron as a cofactor, facilitate the conversion of 5mC to 5hmC.[6] Additionally, iron is an essential nutrient during pregnancy and maternal iron deficiency can leading to lasting neurological conditions in children[6] or an increased risk for preterm pregnancy.[7]

Nutrients involved in OCM are particularly crucial for fetal development. Folic acid supplementation during pregnancy reduces the risk of neural tube defects.[8] Some studies have shown that maternal B_{12}, betaine, and folate intake are inversely correlated with cord blood global DNA methylation.[9,10] Other studies have shown that prenatal folate intake has a positive association with global DNA methylation in cord blood.[11] However, few studies have examined the relationship between DNA 5hmC and nutrients.

In order to better understand relationships between prenatal nutrients involved in OCM and epigenetic programming during sensitive prenatal windows, we aimed to evaluate the extent to which prenatal trimester-specific intake of vitamins B_2, B_6, B_{12}, folate, betaine, choline, methionine, iron, and zinc are associated with global 5mC and 5hmC in cord blood nucleated cells, and if relationships between prenatal maternal nutrient intake and 5mC or 5hmC persist in early (~3 years) and mid-childhood (~7 years) leukocytes. Understanding the relationship between prenatal trimester-specific methyl donor intake and fetal epigenetic programming and its persistence can provide insight on how diet and nutrient supplementation play a role in early-life programming.

Methods

Study population

We leveraged data and samples from Project Viva, a prospective pre-birth cohort study of pregnant mothers recruited between 1999 and 2002, as previously described.[12] Study participants were recruited during the mothers' first prenatal visit at Atrius Harvard Vanguard Medical Associates, a multispecialty medical group practice in Massachusetts, United States. Eligibility criteria included fluency in English, gestational age less than 22 weeks at the first prenatal visit, and singleton pregnancy. From 2128 live births, cord blood was collected from 1018 infants born at one of the two study hospitals. We quantified global genomic 5hmC and 5mC content in 434 cord blood samples for mothers who provided consent for genetic analysis. We evaluated persistence of cord blood associations in 108 children with available blood epigenetic measurements in early childhood (age range: 2.9–4.9 years) and 390 children with blood epigenetic measurements in mid-childhood (age range: 6.7–10.5 years) with complete exposure and covariate information. Mothers provided written informed consent at recruitment and at postpartum follow-up visits. The Institutional Review Board of Harvard Pilgrim Health Care reviewed and approved all study protocols.

Micronutrient estimation from food frequency questionnaires

During the first- and second-trimester study visits we administered a semi-quantitative food frequency questionnaire (FFQ) modified from a validated instrument used in the Nurses' Health Study and other cohorts.[13,14] The FFQ was calibrated for use in pregnancy and nutrient intake was energy-adjusted accordingly.[10,15–17] In the first-trimester visit, a 33-item detailed interview about use (frequency, brand/type, dosage, and timing) of nutritional supplements including vitamin B_{12} and folic acid in early pregnancy was also used to estimate nutrient intake. The second-trimester FFQ included questions about use of nutritional supplements including vitamin B_{12} and folic acid, used to calculate second-trimester intake of these nutrients. Total maternal intake of nutrients periconceptionally was estimate by summing both food and supplement contributions. Briefly, this is done by multiplying a weight assigned to the frequency of use by the nutrient composition for the portion size specified for each food or vitamin supplement reported by the participant. The contribution is then summed to intake across all foods and supplements to obtain a total nutrient for each participant at specific trimesters.[17]

Previous research has shown the methyl donor nutrients to be validly measured by similar FFQs.[15,16] Micronutrient intake was energy-adjusted using a residual model.[18] Data from the FFQ and interviews with mothers indicated that this was a generally folate-replete population.[10] In addition, mothers self-reported sociodemographic characteristics on questionnaires during prenatal visits (age at enrollment, smoking during pregnancy, fish consumption, marital status, education, and race/ethnicity) and child's sex.

Measurement of DNA methylation and 5-hydroxymethylation

Global epigenetic measures were quantified according to the procedures described previously.[19] Briefly, individual deoxyribonucleosides were obtained through a one-step procedure where DNA was isolated from buffy coat and enzymatically hydrolyzed. Following hydrolysis, 490 µL ACN (acetonitrile): H_2O (90:8, v/v) was added to each sample. Calibration standards were created for 5mC, 5hmC, and cytosine (C) by dissolving commercial standards (Sigma-Aldrich) in liquid chromatography grade H_2O. Global genomic measures of 5mC and 5hmC were measured simultaneously using ultrapressure liquid chromatography in tandem with mass spectrometry, as previously described.[20] The lower limits of detection were 0.096 ng/mL, 0.008 ng/mL, and 0.62 ng/mL for 5mC, 5hmC, and C, respectively. The range of accuracy for the concentrations tested varied between 87% and 114.8% of the target. Additionally, precision expressed as a relative standard error ranged from 0.8% to 12.1% with respect to this assay. The percent 5mC and 5hmC was calculated by dividing the appropriate epigenetic modification by the sum of 5mC, 5hmC, and C. A total of 24 samples (11 in cord blood, 4 in early, and 9 in mid-childhood) were below the limit of quantification and excluded from subsequent analyses.

Statistical Analyses

We used means and standard deviations (SD) for continuous measurements and frequencies and proportions for categorical covariates to describe our study sample. Pearson's correlations were

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calculated between first- and second-trimester nutrient intake and %5mC and %5hmC to examine covariation across trimesters as we sought to examine trimester-specific associations. Two different multivariate linear regression models were used to test associations. Each model had either %5mC or %5hmC as the outcome. Cell type and child sex were controlled for in all models to act as precision variables as these variables can have an effect on global epigenetic measures.[21] All models included maternal education, marital status, and race/ethnicity which we considered to be proxies for socioeconomic status and social stressors. These sociodemographic variables may be associated with diet and previous studies have shown how socioeconomic status can be related to global epigenetic measures, so we considered them as confounders. For example, in this study cohort, maternal marital status and education have been associated with %5mC or %5hmC at birth or in childhood.[22] Self-identified race/ethnicity was included as proxy for structural racism. Sensitivity analyses were performed using fish intake, as fish contains methyl donors that are part of our exposure of interest and other nutrients that may play a role in mediating epigenetic programming. For example, fish contains docosahexaenoic acid (DHA), omega-3 and omega-6 long-chain polyunsaturated acids, choline, iodine, iron, and mercury.[23] There is evidence that DHA, omega-3 and mercury play a role in epigenetic programming during pregnancy.[19,24] However, there was insufficient evidence to suggest that fish intake contributed to our model, to avoid overfitting our models, fish intake was not included in our final models. Furthermore, we included maternal age at enrollment and smoking status during pregnancy as we hypothesized they would impact both diet and global measures of epigenetic modification.[25,26]

The first model (model 1) assessed associations with first-trimester nutrient intake of B_2, B_6, B_{12}, folate, betaine, choline, methionine, iron, or zinc separately while adjusting for potential confounders and precision variables. Confounders were validated for each model using Ridge and Lasso regression. The second model (model 2) included separate models for each nutrient to assess the main effect of second-trimester nutrient intake adjusting for first-trimester intake to adjust for time-based confounding, while adjusting for the same previous confounders. All models were adjusted for the demographic and behavioral variables detailed above, and for estimated nucleated cell-type composition (B-cell, CD4T, CD8T, granulocytes, monocytes, and natural killer cells and in cord blood models we included nucleated red blood cell proportions). The distributions of the residuals of the outcomes were assessed for normality using the Shapiro–Wilk's test.

We estimated nucleated cell-type composition from high dimensional DNA methylation data (Illumina 450K BeadChip Arrays) for cord blood and at early and mid-childhood using the Houseman regression calibration method and for cord blood the Bakulski reference panel.[21,27] Briefly, this was accomplished by leveraging cell-type specificity of CpGs from nucleated cells as measured by genome-wide DNA methylation arrays and using reference panels of nucleated cells.[19] Similar models were run for early and mid-childhood with the replacement of %5mC and %5hmC and estimated cell-type with respect to the specific age when samples and measurements were conducted. We report regression estimates and unadjusted 95% confidence intervals (95% CIs) to evaluate precision. Statistical analyses were performed using *R* version 3.3.0 (www.r-project.org).[28]

Table 1. Participant characteristics among 434 mother–child pairs with cord blood epigenetic data from Project Viva

Participant characteristics	*N* (%) or mean (SD)
Child sex – male	226 (52.1%)
Maternal smoking during pregnancy	44 (10.1%)
Maternal age of enrollment (years)	32.3 (5.1)
Mother's education – college graduate	299 (68.9%)
Married or cohabiting	403 (92.9%)
Mother's race/ethnicity	
White	325 (74.9%)
Asian	19 (4.4%)
Black	42 (9.7%)
Hispanic	28 (6.5%)
Other	20 (4.6%)
Cord blood %5mC	5.62 (2.04)
Cord blood %5hmC	0.25 (0.15)
Early childhood %5mC[a]	5.05 (1.73)
Early childhood %5hmC[a]	0.17 (0.11)
Mid-childhood %5mC[b]	5.16 (1.90)
Mid-childhood %5hmC[b]	0.19 (0.11)

[a]$n = 108$.
[b]$n = 390$.

Results

Participant characteristics

A total of 434 mother–child pairs were included in analyses. Approximately half (52.1%) of the children were male. The mean maternal age at enrollment was 32.3 years, 74.9% of mothers were white, 9.7% Black, 4.4% Asian, 6.5% Hispanic and 4.6% identified as another race or ethnicity, 68.9% were college graduates, and 10.1% reported smoking during pregnancy. Mean (SD) of global cord blood %5mC was 5.62% (2.04) and %5hmC was 0.25% (0.15) (Table 1). There was complete data on global epigenomic biomarkers and prenatal first-trimester nutrients among 434, 108 and 390 participants for cord blood, early- and mid-childhood models. While for second-trimester nutrients we had information on 391, 95 and 348 participants in cord blood, early and mid-childhood (supplemental Figure 1).

Associations with *a priori* selected sociodemographic variables, cell-type composition and global cord blood epigenetic biomarkers are shown in supplemental Table 1. Maternal education, marital status, race/ethnicity, and cell-type composition were significantly associated with global epigenetic measurements (Supplemental Table 1). Associations between global epigenetic measures and other sociodemographic variables have been previously descried in this cohort.[22]

Mean (SD) of first- and second-trimester-specific intake for micronutrients tested are reported in Table 2. While some first-trimester nutrient intake values were significantly positively correlated with second-trimester intake, like methionine ($r = 0.53$), the majority of first- and second-trimester nutrient intake were not highly correlated. Additionally, global cord blood %5hmC was negatively correlated with first-trimester B_{12} intake ($r = -0.11$ $p = 0.03$) while %5mC was not significantly correlated with any

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Table 2. Trimester-specific nutrient intake among 434 mother–child pairs with cord blood epigenetic data from Project Viva

	First trimester ($N = 434$)	Second trimester ($N = 391$)
	Mean (SD)	
Fish consumption (serving/day)	0.24 (0.20)	0.22 (0.18)
B_2 intake (mg)	3.78 (3.55)	3.90 (1.40)
B_6 intake (mg)	4.85 (5.75)	5.38 (6.30)
B_{12} intake (μg)	10.3 (6.3)	10.3 (4.5)
Folate intake (mg)	0.94 (0.39)	1.25 (0.39)
Betaine intake (g)	0.23 (0.10)	0.23 (0.10)
Choline intake (g)	0.33 (0.07)	0.32 (0.07)
Methionine intake (g)	2.03 (0.41)	2.05 (0.38)
Iron intake (mg)	33.3 (16.6)	48.4 (25.4)
Zinc intake (mg)	25.6 (10.4)	34.5 (10.6)

estimated nutrient among 391 participants with complete data in all micronutrients (Fig. 1). Unadjusted correlations for all nutrients and epigenetic biomarkers, and their measurements at different life stages are shown in Figure 1.

The relationships of first ($n = 434$) and second ($n = 391$)-trimester nutrient intake with epigenetic outcomes was tested in cord blood at birth and persistence was evaluated at subsequent ages. Data for early childhood (approximately three years of age) and mid-childhood (approximately seven years of age) were available for 108 and 390 children, respectively and the number of participants that were sampled across participants across all three timepoints is shown in supplementary Figure 1. Unadjusted associations between cord blood epigenetic biomarkers and methyl donors by trimester are shown in supplementary Table 2. Associations from fully adjusted linear regression models between prenatal trimester-specific nutrient intake and %5mC and %5hmC in cord blood are presented in Table 3. Results for associations with the %5mC and %5hmC in early and mid-childhood for any significant nutrient are shown in Table 4. We summarize findings for the trimester-specific nutrients and epigenetic modifications below.

Associations with B_{12}

There was a significant inverse association between prenatal maternal intake of B_{12} during the first trimester and cord blood %5hmC with each μg increase associated with a 0.002 lower % 5hmC (95% CI: −0.005, −0.0003) in cord blood. This association was persistent into early childhood ($\beta = -0.007$; 95% CI: −0.01, −0.001). However, there was no evidence of persistence in mid-childhood ($\beta = 0.0003$; 95% CI: −0.002, 0.002). Unadjusted scatterplots of this association are shown in supplemental Figure 2. Bivariate plots must be interpreted considering un-adjustment for major determinants of epigenomic biomarkers like cell-type composition.

Associations with iron

A significant association was found between greater second-trimester iron intake and lower %5mC in cord blood ($\beta = -0.01$. 95% CI: −0.02, −0.002), and this association did not persist into early or mid-childhood. Second-trimester iron intake was also associated with lower %5hmC in cord blood ($\beta = -0.001$. 95% CI: −0.001, −0.00001).

Associations with folate

There were no significant associations between folate intake and any of the epigenetic measures in cord blood. However, a significant positive association between maternal folate consumption during the second-trimester of pregnancy and %5hmC ($\beta = 0.08$; 95% CI: 0.003, 0.16) was observed in in early childhood, but the association was attenuated in mid-childhood ($\beta = 0.03$; 95% CI: −0.002, 0.06).

Associations with zinc

Second-trimester zinc intake was associated with 0.003 greater %5hmC (95% CI: 0.0004, 0.006) in early childhood but this association was attenuated in mid-childhood ($\beta = 0.001$; 95% CI: −0.0001, 0.002).

Discussion

We investigated associations between prenatal trimester-specific intake of micronutrients and global genomic %5mC and %5hmC in blood cells collected at birth and in childhood. We observed that higher estimated B_{12} intake during the first trimester of pregnancy was associated with lower global %5hmC in cord blood. There was evidence of this association persisting in early but not mid-childhood. Second-trimester iron was associated with lower %5mC and %5hmC in cord blood but not in early but not mid-childhood. Although second-trimester folate intake was not associated with %5hmC or %5mC in cord blood, we observed a positive association with %5hmC in early childhood and attenuation of this relationship in mid-childhood. Finally, zinc was associated with greater %5hmC in early childhood only.

Previous studies have tested associations with a limited number of micronutrients and selected global measures of epigenetic variation. For example, a cohort study based in Canada ($n = 288$) applied similar methods to test associations between micronutrient levels in pregnancy and global %5mC and %5hmC in cord blood. High-pressure liquid chromatography was used quantify %5mC and %5hmC and nutrients were assessed using blood serum levels. However, the influence of cell-type composition was controlled for by adjusting for only lymphocyte count whereas in our study we adjusted for cell-type composition. Unlike our study, that study reported that folate serum levels at study recruitment were negatively associated with %5mC in cord blood.[8] The same study also found a negative association between folate levels in maternal red blood cells and %5hmC[8] and a significant positive association between betaine and %5hmC.

Research from the WATCH cohort study in Australia reported no association between child B_2 intake and global %5mC in cord blood, whereas maternal nutrient intake was not reported only adjusted for during pregnancy.[29] Conversely, in our study there was a marginal negative association between maternal prenatal B_2 intake and %5mC in cord blood and positive association with %5hmC in early childhood. The WATCH study was conducted using buccal cells and blood and measured %5mC through capture and detection antibodies for 5mC[29] potentially explaining differences in findings. To our knowledge, no studies have investigated the relationships of maternal B_2 intake during pregnancy and %5hmC in childhood.

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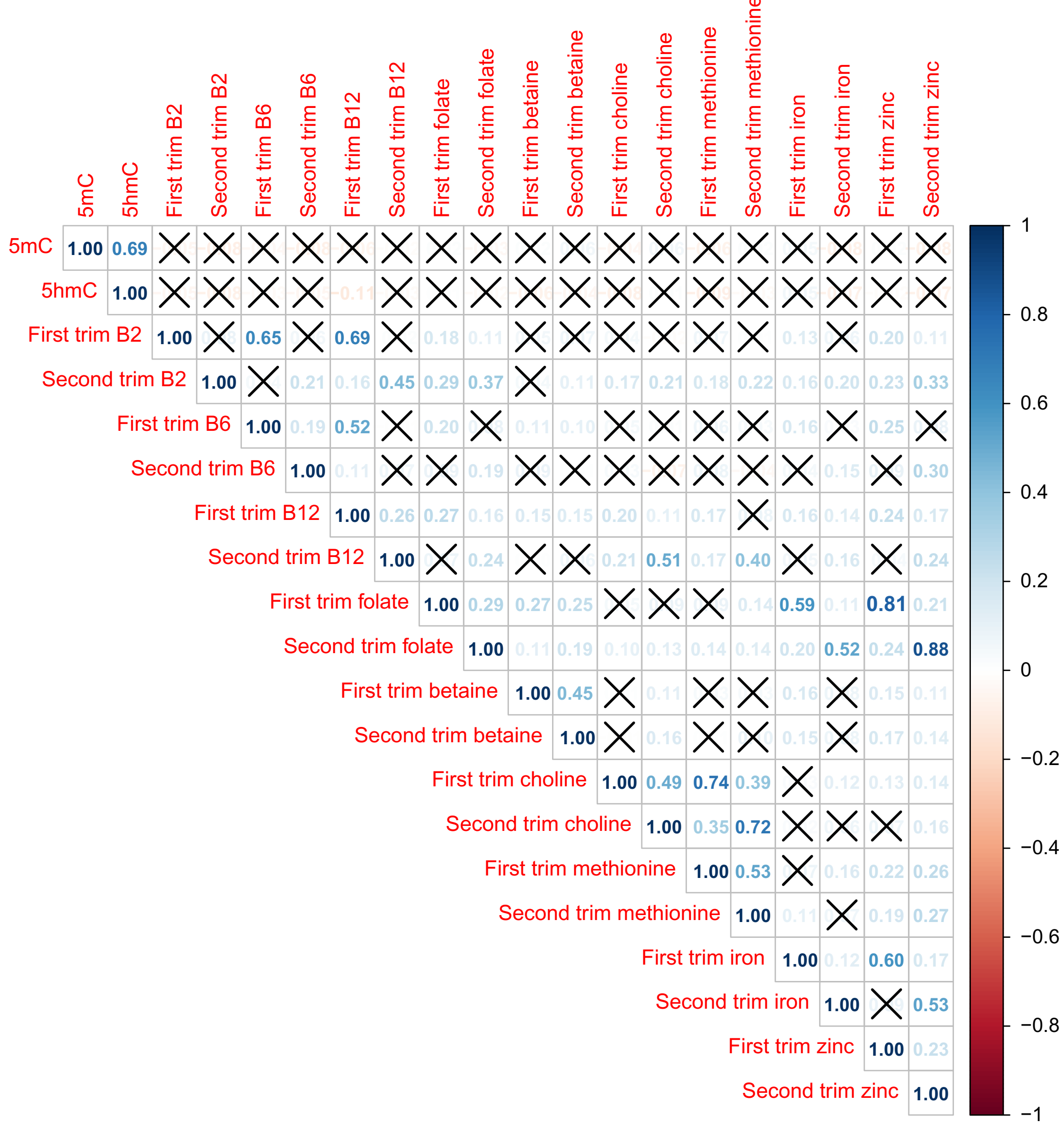

Figure 1. Correlogram of prenatal nutrient intake and global measures of cord blood DNA methylation and hydroxymethylation among $n = 391$ participants with complete nutrient and epigenetic data. Boxes with X are for nonsignificant associations; $p > 0.05$.

A nested cohort study in the North Cumbria Community Genetics Project, in England, used a LUMA assays to determine total %5mC and found a negative association between B_{12} serum levels and %5mC in cord blood.[9] Prior research in Project Viva evaluating LINE-1 methylation found no association between maternal B_{12} intake and %5mC in cord blood.[10] B_{12} is difficult to absorb as the absorption process is mediated by intrinsic factor binding, as a result the bioavailability of B_{12} is likely lower than reported by the FFQ.[30] The relationship we observed between B_{12} and %5hmC could be more pronounced if serum levels of B_{12} were used instead of an FFQ, as an FFQ is only a proxy for fetal exposure to micronutrients.

Our study's findings are unique in that we observed that second-trimester iron intake was associated with increased %5mC and lower %5hmC in cord blood. While most of our participants had iron levels that exceeded the minimal levels recommended by IOM suggestions for iron consumption during pregnancy (27 mg), there were 78 (15.8%) participants in our study who had iron intake lower than these guidelines.[31] Iron intake might be linked with the conversion of 5mC to 5hmC through TET enzymes, which are iron-dependent hydroxylases.[6]

We observed an association between folate and %5hmC in early childhood, although we did not observe associations in cord blood. Since Project Viva is a folate-replete population, there might be insufficient variation to detect associations eliciting an epigenetic response during fetal development.[10] The observed changes are relatively small in magnitude, compared to means and SDs, which was expected as these are global epigenomic measures across the genome. Effect sizes at specific genomic locations and genes might be much greater or smaller with differential implications for expression and health outcomes that need to be further investigated. There is insufficient evidence to suggest that prenatal maternal folate can continue to impact child development after birth via fetal epigenetic reprogramming. Furthermore, folate deficiency has been linked to decreased DNA methylation of CpG sites and certain genes. Decreased DNA methylation of CpG sites can enhance gene transcription, increase DNA strand breakage, which can result in mutations or apoptosis.[11,32] The inconsistency of

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Table 3. Adjusted associations of trimester-specific nutrient intake with global cord blood DNA methylation (%5mC) and 5-hydroxymethylation (%5hmC)

	Model 1: First-trimester nutrients[a]		Model 2: Second-trimester nutrients[b]	
	$N = 434$		$N = 391$	
	Change in %5mC (95% CI)	Change in %5hmC (95% CI)	Change in %5mC (95% CI)	Change in %5hmC (95% CI)
B_2 (mg)	−0.04 (−0.09, 0.02)	−0.002 (−0.006, 0.002)	−0.13 (−0.28, 0.01)	−0.007 (−0.017, 0.004)
B_6 (mg)	−0.01 (−0.05, 0.02)	−0.001 (−0.003, 0.002)	−0.03 (−0.06, 0.01)	−0.001 (−0.004, 0.001)
B_{12} (μg)	−0.02 (−0.05, 0.01)	**−0.002 (−0.005, −0.0003)**	−0.01 (−0.05, 0.04)	0.000 (−0.003, 0.004)
Folate (mg)	0.28 (−0.25, 0.81)	0.024 (−0.014, 0.062)	−0.31 (−0.85, 0.23)	−0.013 (−0.053, 0.026)
Betaine (g)	0.82 (−1.13, 2.76)	−0.056 (−0.196, 0.083)	1.17 (−1.12, 3.47)	−0.019 (−0.186, 0.149)
Choline (g)	−0.96 (−3.93, 2.01)	−0.125 (−0.337, 0.088)	3.09 (−0.43, 6.61)	0.163 (−0.093, 0.420)
Methionine (g)	−0.20 (−0.67, 0.27)	−0.023 (−0.057, 0.010)	0.34 (−0.29, 0.97)	0.012 (−0.034, 0.058)
Iron (mg)	0.01 (0.00, 0.02)	0.001 (0.000, 0.002)	**−0.01 (−0.02, −0.002)**	**−0.001 (−0.001, −0.00001)**
Zinc (mg)	0.01 (−0.01, 0.03)	0.0004 (−0.001, 0.002)	−0.02 (−0.04, 0.00)	−0.001 (−0.002, 0.0003)

Bolded results achieved statistical significance at a cutoff of $p < 0.05$.
[a]Model 1: Each first-trimester nutrient intake was modeled independently adjusting for maternal age at enrollment, education, marital status, race/ethnicity, pregnancy smoking status, infant sex, and cell-type composition.
[b]Model 2: Each second-trimester nutrient intake was modeled independently adjusting for first-trimester intake of the respective nutrient, maternal age at enrollment, education, marital status, race/ethnicity, pregnancy smoking status, infant sex, and cell-type composition.

evidence across timepoints might be due to measurement error or insufficient variation in micronutrient intake and must be cautiously interpreted. Few other studies have investigated the relationship between zinc and %5hmC. No relationship between zinc and LINE-1 methylation was observed in previous Project Viva studies.[10] A previous study reported that Zinc modified association between cadmium and greater DNA methylation of the imprinted gene (*PEG3*) in cord blood.[33]

To understand how the findings of this study compare with the existing literature, it is necessary to consider the methodological differences between studies. The methods of measuring nutrient levels (e.g., the time of pregnancy, estimating nutrient intake or measuring serum levels) may contribute to variations in results, as serum levels may not reflect dietary intake of micronutrients. Considering the sensitive window of epigenetic programming, measuring nutrient intake at different times may alter the direction of the association depending on the epigenetic processes that are underway during the time of collection. Methodological differences in measuring %5mC and %5hmC can also contribute to variations in results. For example, ultra-pressure or high-pressure liquid chromatography results might differ substantially from studies that reported %5mC using LINE-1 repetitive elements, as LINE-1 is a non-coding region and may not reflect global genomic changes in 5mC and 5hmC seen in coding and non-coding regions. In addition, LUMA assays may differ from high-pressure liquid chromatography because the success of a DNA-digestion-based assay is dependent on the integrity of the DNA.[34] Additionally, epigenetic measures may be affected by differences in cell-type distribution of samples or tissue types assayed across studies which are not adjusted for in most studies.[21]

One of the strengths of the current study was the evaluation of sensitive windows of micronutrient exposure and %5mC and % 5hmC at multiple time points to assess persistence of associations in childhood. Furthermore, as the food frequency questionnaires were administered prior to the samples being taken for %5mC and %5hC, temporality can be established. An additional strength of this study is the adjustment for cell-type composition variation.

An important limitation was that micronutrient levels were assessed via food frequency questionnaires and as a result, they may not reflect maternal bioavailability of nutrients. Specifically, while the FFQ utilize has been calibrated for pregnant people the specific performance across methyl donors has not been evaluated. Additionally, genetic variation influencing the availability of methyl donors could also generate inter-individual variation on estimated levels. Future research should consider the influence of genetic polymorphisms for this associations. Using biomarkers of prenatal exposure like serum or red cells for methyl donors' quantification could also improve any exposure misclassification. In addition, we conducted a large number of analyses for numerous nutrients, using two different pregnancy periods, and child global DNA methylation outcomes at multiple time points. However, we did not adjust for multiple testing, thus our findings should be interpreted with caution, especially at age three due to small sample size. Using a post hoc false discovery rate adjustment of 5%, none of the associations survived multiple testing adjustment. Associations due by chance are possible so we report effect sizes and measures of uncertainty (95% CIs) so findings can be compared in future studies. While we expected to find consistent results between %5hmC and %5mC for the same nutrient, this was not the case for our findings. These kinds of discrepancies may exist due to measurement error, or a potential finding may be obscured due to the fact that %5mC and %5hmC represent the global epigenetic measures, averaged across multiple cell types and genomic regions. Across models, %5hmC tended to have stronger evidence of associations with nutrients compared to %5mC, suggesting that % 5hmC might be the more sensitive global measure of early-life epigenetic programming. While changes in global %5mC and %5hmC serve as one measure of epigenetic programming, we did not analyze gene-specific information on %5mC or %5hmC. Global measures do not capture the more nuanced changes in differential methylation at biologically relevant genes.

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Table 4. Adjusted regression coefficients for the persistence of associations between prenatal maternal first or second-trimester nutrient intake and DNA methylation (%5mC) and 5-hydroxymethylation (%5hmC) in early (~3 years) and mid-childhood (~7 years)

	Model 1: First-trimester nutrients[a]		Model 2: Second-trimester nutrients[b]	
	Change (95% CI)		Change (95% CI)	
	%5mC	%5hmC	%5mC	%5hmC
Early childhood	***N* = 108**	***N* = 108**	***N* = 95**	***N* = 95**
B_2 (mg)	−0.22 (−0.63, 0.20)	−0.023 (−0.050, 0.005)	0.09 (−0.46, 0.64)	0.038 (−0.00001, 0.075)
B_6 (mg)	0.10 (−0.06, 0.25)	0.008 (−0.003, 0.018)	0.19 (−0.07, 0.45)	0.010 (−0.008, 0.029)
B_{12} (μg)	−0.06 (−0.16, 0.04)	**−0.007 (−0.014, −0.001)**	−0.02 (−0.13, 0.10)	0.003 (−0.006, 0.011)
Folate (mg)	−0.36 (−1.42, 0.70)	−0.040 (−0.112, 0.032)	0.28 (−0.84, 1.40)	**0.08 (0.003, 0.157)**
Betaine (g)	0.73 (−2.88, 4.35)	−0.070 (−0.316, 0.176)	0.69 (−3.40, 4.78)	0.074 (−0.218, 0.366)
Choline (g)	−1.75 (−7.16, 3.67)	−0.108 (−0.477, 0.261)	−3.38 (−13.0, 6.24)	−0.279 (−0.957, 0.400)
Methionine (g)	−0.64 (−1.45, 0.16)	−0.030 (−0.086, 0.025)	0.03 (−1.34, 1.41)	0.042 (−0.055, 0.139)
Iron (mg)	−0.001 (−0.03, 0.03)	−0.0002 (−0.002, 0.002)	−0.01 (−0.03, 0.01)	−0.0002 (−0.002, 0.001)
Zinc (mg)	−0.02 (−0.05, 0.01)	−0.001 (−0.004, 0.001)	0.02 (−0.02, 0.06)	**0.003 (0.0004, 0.006)**
	%5mC	**%5hmC**	**%5mC**	**%5hmC**
Mid-childhood	***N* = 390**	***N* = 390**	***N* = 348**	***N* = 348**
B_2 (mg)	−0.002 (−0.05, 0.05)	0.000 (−0.003, 0.003)	−0.04 (−0.15, 0.07)	0.002 (−0.005, 0.008)
B_6 (mg)	−0.01 (−0.04, 0.03)	0.000 (−0.002, 0.002)	−0.0002 (−0.03, 0.03)	0.000 (−0.001, 0.001)
B_{12} (μg)	−0.01 (−0.04, 0.02)	0.0003 (−0.002, 0.002)	−0.01 (−0.03, 0.01)	0.000 (−0.001, 0.001)
Folate (mg)	0.37 (−0.15, 0.89)	0.010 (−0.021, 0.040)	−0.02 (−0.58, 0.54)	0.030 (−0.002, 0.063)
Betaine (g)	1.02 (−0.97, 3.02)	−0.002 (−0.121, 0.118)	−0.55 (−3.22, 2.12)	−0.050 (−0.207, 0.107)
Choline (g)	1.03 (−2.08, 4.13)	0.085 (−0.100, 0.270)	−0.32 (−4.07, 3.43)	−0.064 (−0.284, 0.156)
Methionine (g)	0.12 (−0.35, 0.59)	0.013 (−0.015, 0.041)	0.03 (−0.60, 0.65)	−0.013 (−0.049, 0.024)
Iron (mg)	0.01 (−0.004, 0.02)	0.0003 (−0.0004, 0.001)	0.001 (−0.01, 0.01)	0.0002 (−0.0004, 0.001)
Zinc (mg)	0.01 (−0.01, 0.03)	0.000 (−0.001, 0.001)	−0.001 (−0.02, 0.02)	0.001 (−0.0001, 0.002)

Bolded results achieved statistical significance at a cutoff of $p < 0.05$.
[a]Model 1: Each first-trimester nutrient intake was modeled independently adjusting for maternal age at enrollment, education, marital status, race/ethnicity, pregnancy smoking status, infant sex, and cell-type composition in early and mid-childhood, respectively.
[b]Model 2: Each second-trimester nutrient intake was modeled independently adjusting for first-trimester intake of the respective nutrient, maternal age at enrollment, education, marital status, race/ethnicity, pregnancy smoking status, infant sex, and cell-type composition in early and mid-childhood, respectively.

Conclusion

This observational prospective study demonstrates that maternal intake of specific micronutrients such as B_{12} and iron influence global %5mC and %5hmC in cord blood with B_{12} persisting into early but not mid-childhood. Both second-trimester folate and zinc were positively associated with global %5hmC. While these associations were relatively small, OCM is a highly regulated process and therefore we do not expect to see dramatic changes in global epigenetic measures across the genome. As changes to diet might not lead to large changes in these highly regulated epigenetic processes during development. Further research is needed determine the biological implications and long-term health effects linked to these micronutrients, their impact on epigenetic modifications and long-term diseases susceptibility. Additional studies are necessary to best understand the impacts of dietary changes on long-term child health and what optimal dosages of micronutrients are necessary to prevent adverse health outcomes and provide optimal programming of the fetal epigenome.

Supplementary materials. For supplementary material for this article, please visit https://doi.org/10.1017/S2040174422000642

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