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Neural Tube Defects and Maternal Biomarkers of Folate, Homocysteine, and Glutathione Metabolism

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Abstract

Background—Alterations in maternal folate and homocysteine metabolism are associated with neural tube defects (NTDs). The role that specific micronutrients and metabolites play in the causal pathway leading to NTDs is not fully understood.

Methods—We conducted a case-control study to investigate the association between NTDs and maternal alterations in plasma micronutrients and metabolites in two metabolic pathways, the methionine remethylation and glutathione transsulfuration. Biomarkers were measured in a population-based sample of women who had NTD-affected pregnancies ($n = 43$) and a control group of women who had a pregnancy unaffected by a birth defect ($n = 160$). Plasma concentrations of folate, Vitamin B₁₂, Vitamin B₆, methionine, S-adenosylmethionine (SAM), s-adenosylhomocysteine (SAH), adenosine, homocysteine, cysteine, and reduced and oxidized glutathione were compared between cases and controls after adjusting for lifestyle and sociodemographic factors.

Results—Women with NTD-affected pregnancies had significantly higher plasma concentrations of SAH (29.12 vs. 23.13 nmol/L, $P = 0.0011$), adenosine (0.323 vs. 0.255 $\mu\text{mol/L}$, $P = 0.0269$), homocysteine (9.40 vs. 7.56 $\mu\text{mol/L}$, $P < 0.001$), and oxidized glutathione (0.379 vs. 0.262 $\mu\text{mol/L}$, $P = 0.0001$), but lower plasma SAM concentration (78.99 vs. 83.16 nmol/L, $P = 0.0172$) than controls. This metabolic profile is consistent with reduced methylation capacity and increased oxidative stress in women with affected pregnancies.

Conclusions—Increased maternal oxidative stress and decreased methylation capacity may contribute to the occurrence of NTDs. Further analysis of relevant genetic and environmental factors is required to define the basis for these observed alterations.

Keywords

Neural tube defects; maternal biomarkers; folate; methionine; homocysteine; glutathione

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BACKGROUND

Recent studies have reported a lower prevalence of neural tube defect (NTDs) in the United States population following national folic acid educational programs and mandatory folic acid fortification (Williams et al. 2005; Williams et al., 2002; Honein et al., 2001). Since these preventive measures have been in place, NTDs, including spina bifida, anencephalus and encephalocele, are observed once in approximately every 1200 births (National Birth Defects Prevention Network, 2004) compared to once in every 900 births prior to these measures (Williams et al., 2002). The biological mechanism by which folic acid protects the developing fetus is not completely understood and continues to be investigated.

Folic acid interacts in both methionine remethylation and glutathione transsulfuration (Figure 1). For the developing embryo, biologically available folic acid comes from the maternal diet, in the form of naturally occurring folate found in foods, and from maternal intake of synthetic folic acid from vitamins and fortified foods. Once metabolized, 5-methyl-tetrahydrofolate (5-methyl-THF) is the predominant form of folate in serum and in tissues. This coenzyme form of folate is an essential methyl donor for the conversion of homocysteine to methionine through vitamin B₁₂-dependent methionine synthase. Five-methyl-THF is replenished by the conversion of 5-10-methyl-THF by 5-10 methylene tetrahydrofolate reductase. In the methionine cycle, homocysteine is remethylated to form methionine, which is activated by ATP to form S-adenosylmethionine (SAM), the primary methyl donor for cellular methyltransferase reactions. Following transmethylation, SAM is converted to S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to adenosine and homocysteine. Folate deficiency or reduced activity of 5-10 methylene tetrahydrofolate reductase (van der Put et al., 1995; Botto and Yang, 2000) or methionine synthase (Harmon et al., 1996) will lead to hyperhomocysteinemia or low methionine.

Elevated plasma homocysteine has been observed in mothers who gave birth to children with NTDs (Stegers-Theunissen et al., 1994; Mills et al., 1995; van der Put et al., 2001; van der Put and Blom, 2000; Kruger et al., 2000). Elevated homocysteine concentration may disrupt embryogenesis through a direct embryotoxic effect (Rosenquist et al., 1996) or through indirect effects such as a disruption of methylation, accumulation of SAH, or an increase in oxidative stress (Welch et al., 1998; Lawrence et al., 2003; Castro et al., 2003; Huang et al., 2001). During transsulfuration homocysteine is irreversibly condensed with serine to cystathionine by the B₆-dependent enzyme cystathionine beta synthase (CBS), and subsequently to cysteine, gamma-glutamylcysteine (GluCys) and ultimately to glutathione (Finkelstein, 1998).

Vitamins can lower total plasma homocysteine (tHcy) levels. Folic acid supplements containing 0.5 to 5 mg of daily synthetic folate can lower tHcy levels by 25% while vitamin B₁₂ supplementation can lower tHcy levels an additional 7% (Anonymous, 1998). Inconsistent results have been reported for the association of low vitamin B₁₂ and NTDs (Mills et al., 1992; Thorand et al., 1996; Kirke et al., 1993; Wright, 1995; Wald et al., 1996; Groenen et al., 2004). Ray and Blom (2003), in their review, found a moderate association between low maternal vitamin B₁₂ plasma concentrations and an increased risk of having an NTD-affected pregnancy. Limited information has been published on the relationship between vitamin B₆ and NTDs in humans. Shaw et al. (1999) reported non-significant odds ratios when they assessed this vitamin B₆-NTD relationship in a population-based case-control study. In an animal experiment, vitamin B₆ deficiency caused NTDs (Davis et al., 1970). Low vitamin B₆ has been implicated in the etiology of other birth defects in humans, including nonsyndromic oral clefts (Wong et al., 1999; van Rooij et al., 2003) and congenital heart defects (Czeizel et al., 2004).

We conducted our study to examine each micronutrient and metabolite component of methionine remethylation and glutathione transsulfuration among women who had NTD-affected pregnancies. We measured plasma concentrations of folate, vitamin B₁₂, pyridoxal 5'-phosphate (vitamin B₆), methionine, SAM, SAH, adenosine, homocysteine, cysteine, reduced glutathione (GSH) and oxidized glutathione (GSSG) and two ratios of SAM: SAH and GSSG: GSH in women with NTD-affected pregnancies and compared those concentrations and ratios to women who had pregnancies unaffected by a birth defect.

METHODS

Study design and participants

Women who had NTD-affected pregnancies were identified through the Arkansas Reproductive Health Monitoring System, a statewide birth defects registry. Inclusion criteria included: 1) Arkansas residency at the time of completion of index pregnancy and at the time of study enrollment; 2) the index pregnancy resulted in a live birth, stillbirth, elective termination, or other fetal loss; 3) pregnancy ended between April 1998 and March 2004; 4) a physician diagnosis of anencephaly, spina bifida or encephalocele; 5) participants spoke English or Spanish; 6) the case and control subjects had completed participation in the National Birth Defect Prevention Study (NBDPS) (Yoon et al., 2001). Pregnancies that were affected by a known single-gene disorder, chromosomal abnormality, or syndrome were excluded. Controls were randomly selected from birth certificates registered at the Arkansas Department of Health with birth dates between June 1998 and August 2004. Control women spoke English or Spanish and their index pregnancy resulted in a live birth unaffected by any birth defect.

Home visits were scheduled by a research nurse who obtained written informed consent and blood samples using routine venipuncture. Equipment needed to chill and centrifuge blood samples were taken on site. Case or control subjects who were pregnant or taking any known folate-antagonist medications at the time of the blood draw were not eligible for the study. The blood draw was only taken among women 6 weeks or greater postpartum.

Covariates

Additional information obtained from in-home interviews conducted by a research nurse or from the NBDPS structured computer-assisted telephone interview included: maternal race, age, educational level, periconceptional or current multivitamin, cigarette, alcohol use, current caffeine intake, and maternal diabetes. Daily intakes of energy and micronutrients from diet, including methionine, folate, vitamin B₆, and vitamin B₁₂ were estimated from 70-item Block 2000 Brief Food Questionnaire (Block Dietary Data Systems, 2000) completed during home visits. Folate intake was calculated in dietary folate equivalents (DFE) by multiplying folate from fortified food by 1.7 and adding the result to natural folate in food (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1999). Micronutrient intakes were adjusted for energy using Willett's residual method (Willett and Stampfer, 1998).

Sample preparation and biomarker measurement

Fasting blood samples were collected into EDTA-Vacutainer tubes and immediately chilled on ice before centrifuging at 4000 × g for 10 minutes at 4°C to obtain blood plasma. Plasma aliquots were transferred into cryostat tubes and stored at -80°C until extraction and HPLC quantification. Plasma folic acid and vitamin B₁₂ concentrations were measured using Quantaphase II[®] radioimmunoassay kit from Bio-Rad Inc (Hercules, CA). Plasma levels of pyridoxal-5'-phosphate (vitamin B₆) was performed using HPLC method developed by Lequeu with slight modification for using coulometric electrochemical detection (Lequeu et

al., 1985). The methods to measure the other biomarkers and metabolites in the folate/homocysteine/glutathione pathway in our study have been described elsewhere (Hobbs et al., 2005a; Hobbs et al., 2005b; James et al., 2004).

Statistical Analysis

Sociodemographic and lifestyle characteristics of cases and controls were compared with Fisher's exact test for categorical variables. The Wilcoxon rank sum test was used to compare caffeine intake and energy-adjusted nutrient intake because of the skewed distribution of these measurements. All plasma biomarkers exhibited positively skewed distributions; therefore, to improve normality, biomarker data was log-transformed (natural log) prior to analysis. Mean log-transformed biomarker concentrations of cases and controls were compared using a Student's *t* test, whereas multiple linear regression was used to adjust these comparisons for age, race, educational level, multivitamin supplement intake, smoking, alcohol consumption, caffeine intake, and maternal diabetes. In addition, the proportion with deficient levels of plasma vitamin concentrations was compared between cases and controls. These deficient levels were defined at <6.8 nmol/L for folate (Herbert and Das, 1994), <30 nmol/L of pyridoxal 5'-phosphate for vitamin B₆ (Leklem and Reynolds, 1988) and <258 pmol/L for vitamin B₁₂ (Lindenbaum et al., 1994; Ronnenberg et al., 2002). Odds ratios and 95% confidence intervals for the association between vitamin deficiency and NTD status were computed using logistic regression. Logistic models were further adjusted for maternal age, race, diabetes, current smoking, alcohol use and multivitamin intake status. Analyses were performed using the SAS statistical package, version 9.1 (SAS Institute, Cary, NC).

RESULTS

As shown in Table 1, of the 43 case mothers and 160 controls included in this study, approximately 67.4% of cases and 60.0% of controls were younger than 30 years with a range from 20 to 44 years old. Caucasians made up 86.0% of cases and 76.9% of controls. Current smoking and alcohol use was more prevalent among cases than controls, but not significantly different (smokers: 23.3% vs. 16.3%; $P=0.3675$, alcohol use: 65.1% vs. 55.0%, $P=0.2979$). Caffeine intake did not vary significantly between cases and controls (20.6 vs. 20.6 mg/day, $P=0.9720$). There was significant difference between the number of case mothers (60.5%) and the number of controls (39.4%) who reported regular multivitamin use at the time of the blood draw, however, these numbers were closer between case and control mothers for periconceptional vitamin supplements intake (72.1% and 60.6% respectively, $P=0.2137$). There was no significant difference in daily dietary folate intake among cases and controls (360.1 vs. 356.7 $\mu\text{g DFE/d}$, $P=0.7861$). The total folate intake from diet plus supplement at the time of the blood draw was significantly higher in case than control subjects (814.9 vs. 436.1 $\mu\text{g DFE/d}$, $P=0.0247$) due to higher intake of vitamin supplements by cases.

Of the 43 case pregnancies, 15 (34.8%) had anencephaly, 22 (51.2%) had spina bifida and 6 had encephalocele (14.0%). Twenty-two (51.2%) NTD cases were live born, 6 (14.0%) were stillborn (14.0%), 13 (30.2%) were electively terminated and 2 (4.6%) were a fetal death less than 20 weeks.

Table 2 presents plasma concentrations of biomarkers and intermediate metabolites involved in methionine remethylation and glutathione transsulfuration. P-values were calculated to compare log-transformed mean concentrations between cases and controls, adjusting for maternal diabetes, lifestyle, and sociodemographic variables. Compared to controls, case mothers had lower mean plasma concentrations of SAM ($P=0.0172$), and higher mean plasma concentrations of SAH ($P=0.0011$), adenosine ($P=0.0269$), homocysteine

($P < 0.0001$), and GSSG ($P < 0.0001$). The SAM: SAH ratio ($P < 0.0001$) and GSSG: GSH redox ratio ($P < 0.0001$) were significantly different between cases and controls.

The mean plasma methionine levels is lower in NTD case mothers than in control mothers (25.25 $\mu\text{mol/L}$ vs. 26.20 $\mu\text{mol/L}$), but the decrease is not statistically significant ($P = 0.1082$). Plasma concentrations of folate, vitamin B₁₂, and vitamin B₆ did not differ significantly between cases and controls. However, there were more cases (34.9%) than controls (20.0%) with low plasma vitamin B₁₂ concentrations ($< 258 \text{ pmol/L}$, adjusted OR: 2.21; 95% CI: 1.01, 4.83). There was no significant difference between the number of cases (39.5%) and the number of controls (30.6%) who were deficient in vitamin B₆ ($< 30 \text{ nmol/L}$, adjusted OR: 1.72; CI: 0.83, 3.59). (Data not shown.)

DISCUSSION

Our findings of increased concentrations of plasma homocysteine, SAH and adenosine and decreased concentration of plasma SAM suggest alterations in methionine remethylation among women with NTD-affected pregnancies. Increased SAH is a potent product inhibitor of cellular methyltransferase, which during organogenesis can alter gene expression, cell differentiation and apoptosis (Ehrlich, 2003; Finnell et al., 2002; Perna et al., 2003). Low methionine and SAM concentrations in combination with increased SAH and adenosine concentrations were shown previously to be associated with reduced cellular methylation capacity (Yi et al., 2000).

We found that lower maternal plasma vitamin B₁₂ concentration was associated with an increased risk for NTDs. The odds of having an NTD-affected pregnancy among women with plasma vitamin B₁₂ concentration $< 258 \text{ pmol/L}$ was 2 times the odds observed among women with higher plasma vitamin B₁₂ concentration. This finding is consistent with the conclusion of a moderate association between low maternal B₁₂ status and the risk of fetal NTDs in Ray and Blom's review (Ray and Blom, 2003). Vitamin B₁₂ is a coenzyme for methionine synthase. Its deficiency can retard methionine synthase activity and influence folate homeostasis, causing a functional folate deficiency in cells and disruption of the biosynthesis pathways that utilize folate as substrate, i.e., purine and thymidine synthesis. Serum vitamin B₁₂ concentration as low as 258 pmol/L has been shown to be a risk factor for neurologic signs and hyperhomocysteinemia (Healton et al., 1991; Lindenbaum et al., 1988; Lindenbaum et al., 1994).

Within the transsulfuration pathway, cases had significantly higher concentrations of GSSG and redox ratio of GSSG: GSH compared to controls. The significant increase in GSSG and GSSG: GSH redox ratio is an indication of oxidative stress (Nemeth et al., 2001; James et al., 2004). Chronic oxidative stress would cause a decrease in methionine and SAM by down regulating redox-sensitive enzymes in the methionine cycle including methionine synthase, betaine homocysteine methyltransferase, and methionine adenosyltransferase.

Evidence for oxidative embryopathy and dysmorphogenesis is based largely on animal studies, in which glutathione depletion and oxidative stress have been strongly implicated in the etiology of multiple birth defects (Ishibashi et al., 1997; Sakamaki et al., 1999). For example, exposure of rats to 20% oxygen during early neurulation significantly increased the incident of NTDs relative to unexposed embryos (Ishibashi et al., 1997). Despite abundant supportive evidence from animal studies, limited human studies have been conducted on the association between birth defects and glutathione-mediated oxidative stress. Recently, Hobbs et al. (2005b) measured metabolic biomarkers of increased oxidative stress in mothers with pregnancies affected by congenital heart defects (CHDs) and indicated an alteration in the delicate balance between oxidative stress and antioxidant

defense mechanisms among women with CHDs-affected pregnancies. Our findings of elevated GSSG and redox ratio of GSSG: GSH among case mothers with NTD-affected pregnancies are consistent with those previously reported results (Hobbs et al., 2005a; Hobbs et al., 2005b).

Important methodological limitations of our study should be considered. The blood obtained to measure biomarkers was collected well after the index pregnancies had ended. The median interval between the end of pregnancy and blood draw for all subjects in our study is 20.7 months ranging from 4 to 63 months with no difference between cases and control. ($p=0.8701$, data not shown). The methodological concern regarding the biologic relevance of conducting a study of maternal folate-related metabolism following adverse pregnancy outcomes have been directly addressed in previous publications (Hobbs et al., 2005a; Munger et al., 2004; Tamura et al., 2005). Several studies have shown that maternal serum vitamin B₁₂ and homocysteine measures taken past 6 week postpartum have been reported to return to preconceptional levels (Walker et al., 1999; Cikot et al., 2001). Previously published clinical and epidemiological studies have revealed disturbances in folate metabolism among non-pregnant women who previously had pregnancies affected by NTDs, orofacial clefts, and CHDs (Van der Put et al., 2001; Wong et al., 1999; Kapusta et al., 1999). Metabolic alterations provide a window through which the interactive impact of genes and environment may be viewed and relevant susceptibility factors identified. The small sample size included in our study may limit the power to detect differences in some biomarkers. For example, animal studies have demonstrated that methionine plays an important role in the normal closure of the rodent neural tube. In our study, mean plasma methionine level was lower in NTD case mothers than in controls, but the decrease is not statistically significant ($P=0.1082$). Due to a limited sample size, we were unable to examine the relationship between specific NTD phenotypes and plasma biomarker concentrations, and to evaluate the impact of multivitamin intake on the observed relationships. The basis for the abnormal metabolic profile observed in our study cannot be defined without further analysis of relevant genetic variants for folate-pathway enzymes, activities of folate receptors, and lifestyle factors. The activities of relevant enzymes in the folate pathway, such as 5–10 methylene tetrahydrofolate reductase, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase, and methionine synthase may be reduced due to genetic variants or nutrient intake or both. Boddie et al. (2000) reported the impaired folate absorption in women with a history of NTD-affected pregnancy. A trend for lower absorption of both the naturally occurring food folate and folic acid in cases over a 48-hour period was observed in their study. Recently, Rothenberg and colleagues (2004) identified autoantibodies against folate receptors on placental membranes among women who had NTD-affected pregnancies. The identified autoantibodies effect could alter folate metabolism making folate less available to the developing embryo. In our study, mean plasma folate concentration was not significantly lower among case subjects compared to controls, but the total folate intake from diet plus supplement was significantly higher in case compared to controls ($P=0.0247$), implying differences between the two groups of women in folate absorption, metabolism or cellular uptake. Our study findings add to the growing body of evidence that shows altered plasma levels of biomarkers and metabolites in women with NTD-affected pregnancies compared to women whose pregnancies were unaffected by any birth defect. Investigating the complex etiology of NTDs requires consideration of both the methionine cycle and transsulfuration pathways. If further investigations with larger study populations replicate results of this study, it would strengthen the argument that methylation capacity and the amount of exposure to chronic oxidative stress are important for neural tube closure. Our study findings may provide new etiologic clues about the glutathione antioxidants defense mechanism of NTDs and novel intervention strategies such as antioxidants for primary prevention.

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Figure 1.

The interactive and interdependent pathways of folate and methionine metabolism as related to the synthesis of glutathione (GSH).

Abbreviations: THF: tetrahydrofolate; MTHFR: methylenetetrahydrofolate reductase; CBS: cystathionine beta synthase; GCL: glutamate-cysteine ligase; GSH: reduced glutathione; GSSG: oxidized glutathione; GSTs: glutathione-S-transferases; GR: glutathione synthase reductase; BHMT: betaine homocysteine methyltransferase; GS: glutathione synthase; CysGly, cysteinylglycine; GluCys, Glutamylcysteine

Table 1

Selected characteristics of participants.

	NTD Cases (N=43)	Controls (N=160)	
	Num (%) ¹	Num (%)	P-value ²
Age			
Less than 30 years	29 (67.4)	96 (60.0)	0.4803
30 or more than	14 (32.6)	64 (40.0)	
Race			
Caucasian	37 (86.0)	123 (76.9)	0.4748
African American	4 (9.3)	26 (16.3)	
Others	2 (4.7)	11 (6.9)	
Education			
Less than high school	4 (9.3)	20 (12.5)	0.3554
Completed high school	14 (32.6)	48 (30.0)	
13–15 years education	16 (37.2)	41 (25.6)	
16 education or higher	9 (20.9)	51 (31.9)	
Diabetes			
No diabetes (of any type)	40 (93.0)	154 (96.3)	0.4031
Gestational diabetes	3 (7.0)	6 (3.7)	
Smoker			
No	33 (76.7)	134 (83.8)	0.3675
Yes	10 (23.3)	26 (16.3)	
Periconceptional smoking			
No	33 (76.7)	130 (81.3)	0.5208
Yes	10 (23.3)	30 (18.8)	
Alcohol use			
No	15 (34.9)	72 (45.0)	0.2979
Yes	28 (65.1)	88 (55.0)	
Periconceptional alcohol use			
No	32 (74.4)	118 (73.8)	1.0000
Yes	11 (25.6)	42 (26.3)	
Daily caffeine intake (mg/d)	20.6 (3.6, 48.8) ³	20.6 (4.4, 53.4)	0.9720
Vitamin supplementation			
No	17 (39.5)	97 (60.6)	0.0157
Yes	26 (60.5)	63 (39.4)	
Periconceptional vitamin supplement			
No	12 (27.9)	62 (38.8)	0.2137
Yes	31 (72.1)	97 (60.6)	
Daily dietary folate intake (µg DFE/d)	360.1 (288.1, 420.0)	356.7 (302.3, 429.6)	0.7861 ⁴
Daily total folate intake (µg DFE/d) ⁵	814.9 (373.5, 1220.0)	436.1 (314.8, 942.4)	0.0247

¹ Percentages may not equal 100 because of missing data or rounding.

²P-value is calculated by Fisher's exact test for categorical variables and by Mann-Whitney U test for continuous variables.

³Median; interquartile ranges in parentheses (all such values)

⁴P-value computed by Mann-Whitney U test using energy-adjusted nutrient intake

⁵Total folate intake $\mu\text{g DFE} = \mu\text{g DFE dietary folate intake} + 2 \mu\text{g} * \text{folic acid from supplement}$

Table 2

Summary statistics for plasma biomarker concentrations, and crude and adjusted p-values for the comparison of log-transformed plasma biomarker concentrations, between NTD cases and controls.

	NTD Cases (N=43)			Controls (N=160)		
	Mean ± SD	Median (Range)		Mean ± SD	Median (Range)	
Methionine (μmol/L)	25.25 ± 5.25	24.54 (15.34 – 38.90)		26.20 ± 4.99	26.07 (17.25 – 43.18)	0.1082
SAM (nmol/L)	78.99 ± 13.91	80.14 (40.44 – 115.40)		83.16 ± 13.27	81.01 (50.54 – 127.08)	0.0172
SAH (nmol/L)	29.12 ± 11.91	27.22 (10.53 – 66.24)		23.13 ± 8.35	22.05 (7.67 – 64.68)	0.0011
SAM: SAH	3.15 ± 1.41	3.01 (1.11 – 7.69)		4.00 ± 1.38	3.95 (1.16 – 9.90)	<.0001
Adenosine (μmol/L)	0.323 ± 0.164	0.263 (0.074 – 0.892)		0.255 ± 0.144	0.226 (0.073 – 0.934)	0.0269
Homocysteine (μmol/L)	9.40 ± 3.02	8.66 (5.24 – 21.82)		7.56 ± 1.68	7.45 (4.13 – 13.33)	<.0001
GSSG (μmol/L)	0.379 ± 0.196	0.328 (0.111 – 0.967)		0.261 ± 0.089	0.262 (0.105 – 0.643)	0.0001
GSH (μmol/L)	7.24 ± 1.67	7.06 (4.27 – 11.73)		7.25 ± 1.57	7.14 (4.11 – 12.15)	0.9469
GSSG: GSH	0.055 ± 0.035	0.044 (0.017 – 0.172)		0.037 ± 0.012	0.035 (0.013 – 0.086)	<.0001
Cysteine (μmol/L)	239.13 ± 27.78	241.70 (178.9 – 301.70)		231.36 ± 21.61	231.10 (170.8 – 293.40)	0.1228
Folate (μmol/L)	26.22 ± 15.19	20.85 (6.66 – 74.66)		26.66 ± 13.06	23.10 (7.50 – 90.91)	0.4841
Vitamin B12 (pmol/L)	337.83 ± 149.18	291.43 (160.40 – 698.70)		368.97 ± 149.78	337.88 (74.31 – 926.68)	0.1771
Vitamin B6 (nmol/L)	36.25 ± 11.29	36.15 (19.20 – 64.70)		36.26 ± 11.35	34.33 (14.90 – 82.76)	0.9808
						0.4107

SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; GSH, reduced glutathione; GSSG, oxidized glutathione;

[†] Two-sample t-test using log-transformed data

[‡] P-value adjusted for age, race, education, smoking, drinking status, vitamin intake, caffeine intake, and maternal diabetes.