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Combined Vitamin B-12 and Balanced Protein-Energy Supplementation Affect Homocysteine Remethylation in the Methionine Cycle in Pregnant South Indian Women of Low Vitamin B-12 Status[1–4]

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Abstract

Background: Low-quality dietary protein intake and vitamin B-12 deficiency could interact to decrease methionine transmethylation and remethylation rates during pregnancy and may affect epigenetic modifications of the fetal genome.

Objective: The objective of this randomized, partially open-labeled intervention trial was to examine the effect of supplemental high-quality protein and vitamin B-12 on third-trimester methionine kinetics in pregnant Indian women with a low vitamin B-12 status.

Methods: Pregnant women with low serum vitamin B-12 concentrations (<200 pmol/L) were randomly assigned to 1 of 3 groups: the first group received balanced protein-energy supplementation of 500 mL milk/d plus a 10-μg vitamin B-12 tablet/d (M+B-12 group; $n = 30$), the second group received milk (500 mL/d) plus a placebo tablet (M+P group; $n = 30$), and the third group received a placebo tablet alone (P group; $n = 33$). Third-trimester fasting plasma amino acid kinetics were measured by infusing 1-^{13}C,methyl-$^{2}H_3$-methionine, ring-$^{2}H_5$-phenylalanine, ring-$^{2}H_4$-tyrosine,1-^{13}C-glycine, and 2,3,3-$^{2}H_3$,^{15}N-serine in a subset of participants. Placental mRNA expression of genes involved in methionine pathways, placental long interspersed nuclear elements 1 (LINE-1) methylation, and promoter methylation levels of vascular endothelial growth factor (*VEGF*) were analyzed.

Results: Remethylation rates in the M+B-12, M+P, and P groups were 5.1 ± 1.7, 4.1 ± 1.0, and, 5.0 ± 1.4 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively ($P = 0.057$), such that the percentage of transmethylation remethylated to methionine tended to be higher in the M+B-12 group (49.5% ± 10.5%) than in the M+P group (42.3% ± 8.4%; $P = 0.053$) but neither differed from the P group (44.2% ± 8.1%; $P > 0.1$). Placental mRNA expression, LINE-1, and *VEGF* promoter methylation did not differ between groups.

Conclusions: Combined vitamin B-12 and balanced protein-energy supplementation increased the homocysteine remethylation rate in late pregnancy. Thus, vitamin B-12 along with balanced protein-energy supplementation is critical for optimal functioning of the methionine cycle in the third trimester of pregnancy in Indian women with low serum vitamin B-12 in early pregnancy. This trial was registered at clinicaltrials.gov as CTRI/2016/01/006578. *J Nutr* 2017;147:1094–103.

Keywords: protein, vitamin B-12, methionine kinetics, plasma amino acids, DNA methylation, pregnancy

Introduction

Although nutritional substrates are required for tissue deposition in the growing fetus, the impact of periconceptional maternal undernutrition on DNA methylation (1) is also important both for its effects on the individual during adulthood as well for as its intergenerational effects. For example, severe periconceptional undernutrition has been associated with a lower DNA methylation of the imprinted gene insulin-like growth factor (*IGF*)[11] II (*IGF2*) 6 decades later (2). The supply of methyl groups in the methionine cycle requires an adequate dietary intake of methionine and other methyl donors such as choline, betaine, serine, and glycine, with the involvement of

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vitamins B-6 and B-12 and riboflavin as cofactors (3). Whereas vitamin B-12 and folate are involved in the remethylation of homocysteine, vitamin B-6 is involved in its transsulfuration to cysteine. There is evidence from a recent study in rural Gambia, a region subjected to dramatic seasonal fluctuations in food accessibility, that maternal diet may affect offspring DNA methylation, because the authors reported that infant DNA methylation was influenced by maternal periconceptional plasma homocysteine, vitamin B-6, and cysteine concentrations (4).

A relative methionine deficiency is likely to reduce the rates of transmethylation and remethylation during pregnancy (5), with a potential impact on both protein synthesis and DNA methylation. As pregnancy progresses, transmethylation and remethylation rates have been shown to increase in pregnant American women (6), but this did not happen in Indian women (7), probably due to the much lower antenatal protein intake of the latter. Equally, vitamin B-12, through its influence on 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), affects the remethylation of homocysteine to regenerate methionine. The coexistence of a poor-quality protein intake in the presence of vitamin B-12 deficiency could have multiplicative effects. Other amino acids may also affect the methionine cycle. Serine and glycine are particularly important as providers of methyl groups for remethylation; serine is also a precursor for glycine synthesis and is involved in cysteine biosynthesis during transsulfuration. Plasma concentrations of serine have been shown to decline during pregnancy (8).

Earlier Indian studies found associations between intrauterine growth retardation and a deficient maternal vitamin B-12 status (9). In a vegetarian diet, milk and other dairy products are the major sources of both high-quality protein and vitamin B-12 (10). Furthermore, milk also acts as an energy-balanced source of protein in the diet, which is important especially during pregnancy because high-protein supplementation has been reported to adversely affect birth outcomes (11, 12). This is relevant in India, where vegetarian diets with low dairy intakes are the norm, and where close to 40% of the burden of low birth weight exists (13). Hence, combined high-quality protein and vitamin B-12 intervention studies are required in India with specific reference to the kinetics of methyl donors and epigenetic and transcriptional changes in critical genes in the placenta.

This study describes a partially open-labeled, randomized controlled trial in pregnant Indian women with low vitamin B-12 status to primarily assess the effect of high-quality protein (milk) and vitamin B-12 supplementation from recruitment (11 ± 2 wk of gestation) until delivery, on the kinetics of methionine and its transmethylation, transsulfuration, and remethylation; serine and glycine fluxes and their interconversions; and phenylalanine kinetics (a marker of whole-body protein turnover) in the third trimester of pregnancy. The effect of this supplementation on the placental expression of selected genes that play a significant role in one-carbon metabolism and DNA methylation and in fetoplacental growth and placental angiogenesis, placental long interspersed nuclear elements 1 (LINE-1) methylation (surrogate for placental global methylation), and promoter methylation levels of vascular endothelial growth factor (*VEGF*) was also evaluated.

Methods

This partially open-labeled, randomized controlled trial was conducted between December 2010 and February 2014 at the St. John's Research Institute and the obstetrics department of St. John's Medical College Hospital, in Bangalore, India, which caters to patients of diverse socioeconomic status. The Institutional Ethical Review Board approved the experimental protocols. A written consent was obtained from each study subject at enrollment. This study was a partially open-label interventional trial (registered at clinicaltrials.gov as CTRI/2016/01/006578).

Subjects and supplements. Pregnant women in the first trimester of pregnancy (11 ± 2 wk of gestation) attending routine antenatal clinic visits were eligible for the study. Women with multiple pregnancies, those with a clinical diagnosis of chronic illness (e.g., type 2 diabetes, hypertension, heart disease, thyroid disease, and epilepsy), and those who tested positive for hepatitis B surface antigen, HIV, or syphilis were excluded. The women were screened for their vitamin B-12 status, and those with low serum vitamin B-12 (<200 pmol/L) concentrations were enrolled into the study by the study investigators and randomly assigned to 1 of 3 parallel groups to receive milk or vitamin B-12 supplements from recruitment until delivery. One group received balanced protein-energy supplementation of 500 mL milk/d plus a 10-μg vitamin B-12 tablet/d (M+B-12 group), a second group received milk (500 mL/d) plus a placebo tablet (M+P group), and a third group received a placebo tablet alone (P group). The milk supplement of 500 mL/d, which provided 320 kcal energy and 16.5 g protein equivalent to a 21% protein-energy ratio (Karnataka Milk Federation), and the vitamin B-12 tablet (10 μg/d) or placebo tablet (similar in size, shape, and color; Excellamed Laboratories Pvt. Ltd.) were allocated according to the randomization list by the study investigators and home-delivered by a social worker 2 times/wk. The milk supplement also provided 2.0 μg vitamin B-12/d, which is equivalent to ~77% of the RDA of 2.5 μg/d for pregnant women as well as 23.5 g carbohydrate and 18.0 g fat (14, 15). The social workers, subjects, and care providers were blinded to the vitamin B-12 and placebo tablets. All of the subjects received routine antenatal dietary counseling. Compliance in taking the supplements was recorded every month on the basis of the counts of unused supplements. Further efforts to improve adherence to the regimen were achieved by methods described by Duggan et al. (16).

Anthropometric measures and habitual food intakes. At recruitment, age and obstetric history of all of the subjects were recorded. Gestational age was confirmed by ultrasound within 2 wk of the initial visit. Height was measured to the nearest 0.1 cm on a calibrated stadiometer. An FFQ was administered at baseline to obtain information on the habitual dietary intake for the preceding 3 mo. The FFQ was adapted from that developed for the urban middle class residing in south India (17). During the course of pregnancy, dietary intakes were measured each week by multiple 24-h recall. A minimum of 3 recalls were available in the second and third trimesters. The recall used recipes and methods that were developed earlier (17). The mean nutrient intake and food group intake per day were computed for each trimester. During each monthly visit, body weight was recorded using a digital weighing balance (Soehnle, Germany) to the nearest 0.1 kg. Gestational weight gain (GWG) per week was calculated at each antenatal visit. Information on maternal morbidity during pregnancy, such as pregnancy-induced hypertension (18), abnormal glucose tolerance test, gestational diabetes (19), and severe anemia (20) was collected. Blood pressure was recorded

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[4] Supplemental Tables 1–3 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[11] Abbreviations used: GLV, green-leafy vegetable; GWG, gestational weight gain; *IGF*, insulin-like growth factor; LINE-1, long interspersed nuclear elements 1; M+B-12, milk + vitamin B-12; ME, metastable epiallele; M+P, milk + placebo; P, placebo; SGA, small for gestational age; *VEGF*, vascular endothelial growth factor.

randomly in duplicate after 10 min of sitting, at each antenatal visit, with an automated blood pressure monitor (HEM-757; OMRON Intelli Sense, Canada). At delivery, neonatal anthropometric measurements along with maternal complications such as premature rupture of membranes were recorded. The placental tissues were collected for gross microscopic, histologic examinations and molecular analysis. Infant birth weight was measured to the nearest 0.01 kg on a digital weighing scale (BWS 101; Phoenix), and length was measured on a standard infantometer to the nearest 0.1 cm. Birth outcomes were categorized as low birth weight (birth weight <2.5 kg), preterm birth for infants born at <37 wk of gestation, and small for gestational age (SGA; birth weight <10th percentile for gestational age) (21).

Stable isotope infusion protocol. A fasted-state stable isotope kinetic study was conducted in a subset of pregnant women in the third trimester of pregnancy. Subjects were admitted to the metabolic ward in the evening and consumed their last habitual meal before 1900. Approximately 3 h later, an intravenous catheter (Jelco, 22G; Medex Medical Ltd.) was inserted into the antecubital vein of 1 arm for the body-weight–specific infusion of stable isotopes, whereas another catheter was inserted into the dorsal vein of the contralateral hand for drawing blood samples and kept patent with a slow saline drip. A warm blanket around the hand, maintained at 60–65°C, was used to arterialize the venous blood. Sterile solutions of $NaH^{13}CO_3$, 1-^{13}C,methyl-$^2H_3$-methionine, ring-$^2H_5$-phenylalanine, 3,3-$^2H_2$-tyrosine, ring-$^2H_4$-tyrosine, 1-^{13}C-glycine, and 2,3,3-$^2H_3$,^{15}N-serine (Cambridge Isotope Laboratories) were prepared in sterile isotonic saline. After basal blood and breath samples, the bicarbonate pool was primed with 3 μmol $NaH^{13}CO_3$/kg followed by primed-continuous intravenous infusions of 1-^{13}C,methyl-$^2H_3$-methionine (prime = 1.5 μmol/kg, infusion rate = 1.5 μmol · kg^{-1} · h^{-1}), $^2H_5$-phenylalanine (prime = 4 μmol/kg, infusion rate = 4 μmol · kg^{-1} · h^{-1}), and $^2H_2$-tyrosine (prime = 1.5 μmol/kg, infusion rate = 1.5 μmol · kg^{-1} · h^{-1}) for 4.5 h. The tyrosine pool was also primed with 0.5 μmol $^2H_4$-tyrosine/kg. A primed-continuous infusion of 1-^{13}C-glycine (prime = 8 μmol/kg, infusion rate = 8 μmol · kg^{-1} · h^{-1}) was started at 3.0 h. Four breath samples were taken between 2.5 and 3.0 h of the infusion (to calculate methionine oxidation, an index of transsulfuration), then a primed-continuous infusion of 1-^{13}C-glycine (prime = 8 μmol/kg, infusion rate = 8 μmol · kg^{-1} · h^{-1}) was started and maintained for 1.5 h. After the glycine infusion was stopped, a primed-continuous infusion of $^2H_3$,^{15}N-serine (prime = 6 μmol/kg, infusion rate = 6 μmol · kg^{-1} · h^{-1}) was started and maintained for 1.5 h. Serial blood and breath samples were collected every 10 min during the last 0.5 h of each isotope infusion period. Total carbon dioxide excretion was measured for 20 min with an open-circuit indirect calorimeter (MetaMax; Cortex). At the end of the experiment, subjects were given breakfast and discharged.

Laboratory analyses. Blood samples collected at recruitment were analyzed for vitamin B-12 concentration by the electrochemiluminescence method (Elecsys 2010; Roche Diagnostics). Quality-control samples pertaining to low, middle, and high ranges of vitamin B-12 were analyzed along with the samples. Intra- and interassay CVs were 0.5% and 2.4%, respectively. Blood samples during the tracer kinetic period were collected into EDTA-coated anticoagulant tubes (Becton Dickinson) and immediately centrifuged at 1200 × *g* for 15 min at 4°C. The plasma was separated and stored at −80°C until analysis. Whole blood was treated with ascorbic acid and stored for whole-blood hemolysate (RBC folate) analysis by chemiluminescence (ADVIA Centaur XP). Commercially available, whole-blood, quality-control samples were used for this assay. Intra- and interassay CVs were 1.9% and 5.2%, respectively.

The plasma amino acid tracer-to-tracee ratios and concentrations of free amino acids were measured by LC coupled with tandem MS. Methionine, phenylalanine, tyrosine, serine, glycine, and alkylated homocysteine were converted into their DANS [5-(dimethylamino)-1-napthalene sulfonamide] derivative and analyzed by selected reaction monitoring on a triple quadrapole mass spectrometer as previously described (22, 23). The ions were analyzed by selected reaction monitoring. The transitions observed were precursor ions *m/z* 383, 384, and 387 to product ion *m/z* 170 for methionine; precursor ions *m/z* 426 and 427 to product ion *m/z* 170 for homocysteine; precursor ions 399 and 404 to product ion *m/z* 170 for phenylalanine; precursor ions 415, 417, and 419 to product ion *m/z* 170 for tyrosine; *m/z* 309, 310, and 311 to product ion *m/z* 170 for glycine; and precursor ions *m/z* 339, 340, and 343 to product ion *m/z* 170 for serine.

Expired breath samples were collected into plain Vacutainers (Becton Dickinson) and stored at room temperature until analysis. Breath samples were analyzed for $^{13}CO_2$ enrichments by monitoring ions at *m/z* ratios of 44 and 45 in isotope ratio mass spectrometry (Delta V Advantage; Thermo Scientific). Plasma homocysteine was measured by GC-MS (GCMS-MSD; Agilent Technologies, Inc.) based on methyl-chloroformate derivatization (24). Quality-control samples for this assay were prepared in-house from pooled plasma. Intra- and interassay CVs were 2.3% and 4.0%, respectively.

Calculations. Total flux (Q) of an amino acid was calculated using the steady state tracer dilution approach:

$$Q = i(\mathrm{Tr}/\mathrm{Tr}_i/\mathrm{Tr}/\mathrm{Tr}_p) \quad (1)$$

where i is the tracer infusion rate (micromoles per kilogram per hour) and $\mathrm{Tr}/\mathrm{Tr}_i$ and $\mathrm{Tr}/\mathrm{Tr}_p$ are the tracer-to-tracee ratios of the infusate and of plasma methionine, glycine, serine, phenylalanine, or tyrosine at plateau. Endogenous flux was calculated by subtracting the tracer infusion from Q.

Methionine kinetics. The di-labeled 1-$^{13}C_1$, methyl-$^2H_3$-methionine kinetics model was used to calculate kinetics parameters as previously described by MacCoss et al. (25). Because homocysteine formed by transmethylation (TM) is either remethylated back to methionine or transsulfurated (TS) to cysteine and α-ketobutyrate,

$$\mathrm{TM} = \mathrm{RM} + \mathrm{TS} \quad (2).$$

Remethylation (RM) was calculated as the difference between the flux of methyl methionine and carboxy methionine, because they would be different by the rate of homocysteine remethylation,

$$\mathrm{RM} = Q_m - Q_c \quad (3).$$

Because α-ketobutyrate is formed from methionine and eventually oxidized to carbon dioxide, methionine oxidation is equivalent to transsulfuration. Methionine oxidation was calculated by using the M+1 isotopomer of homocysteine as the intracellular methionine carboxy moiety tracer-to-tracee ratio. The rate of carbon dioxide production was calculated from carbon dioxide excretion determined by indirect calorimetry:

$$\begin{aligned}&\text{Methionine Oxidation}\,(\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1})\\&= \text{Breath}\,{}^{13}\text{CO}_2\,\text{enrichment} \times R_a\text{CO}_2/\text{Tr}/\text{tr}_{M+1\,\text{Hcy}}\end{aligned} \quad (4)$$

where R_aCO$_2$ is obtained from VCO$_2$/0.78 (VCO$_2$ is the rate of carbon dioxide excreted in the breath), assuming that in the fasted state, 78% of carbon dioxide produced is excreted in the breath.

The rate of conversion of glycine to serine was calculated from the following equation:

$$Q_{\mathrm{Gly}\to\mathrm{Ser}} = Q_{\mathrm{Ser}} \times \mathrm{IE}_{\mathrm{Ser}}/\mathrm{IE}_{\mathrm{Gly}} \quad (5).$$

The rate of conversion of serine to glycine was calculated from the following equation:

$$Q_{\mathrm{Ser}\to\mathrm{Gly}} = Q_{\mathrm{Gly}} \times \mathrm{IE}_{\mathrm{Gly}}/\mathrm{IE}_{\mathrm{Ser}} \quad (6)$$

where, $Q_{\mathrm{Gly}\to\mathrm{Ser}}$ is the total glycine or serine flux, $\mathrm{IE}_{\mathrm{Gly}}$ is the plasma glycine enrichment, and $\mathrm{IE}_{\mathrm{Ser}}$ is the plasma enrichment of serine. Phenylalanine hydroxylation to tyrosine (Q_{PT}; an index of phenylalanine oxidation) and nonoxidative disposal ($\mathrm{Phe}_{\mathrm{syn}}$; an index of protein synthesis) were calculated as previously described by us (26).

Placenta collection and storage. Placentas were collected immediately at birth and fixed in 10% neutral buffered formalin. The trimmed placental weights were measured. Bits from representative parts of the

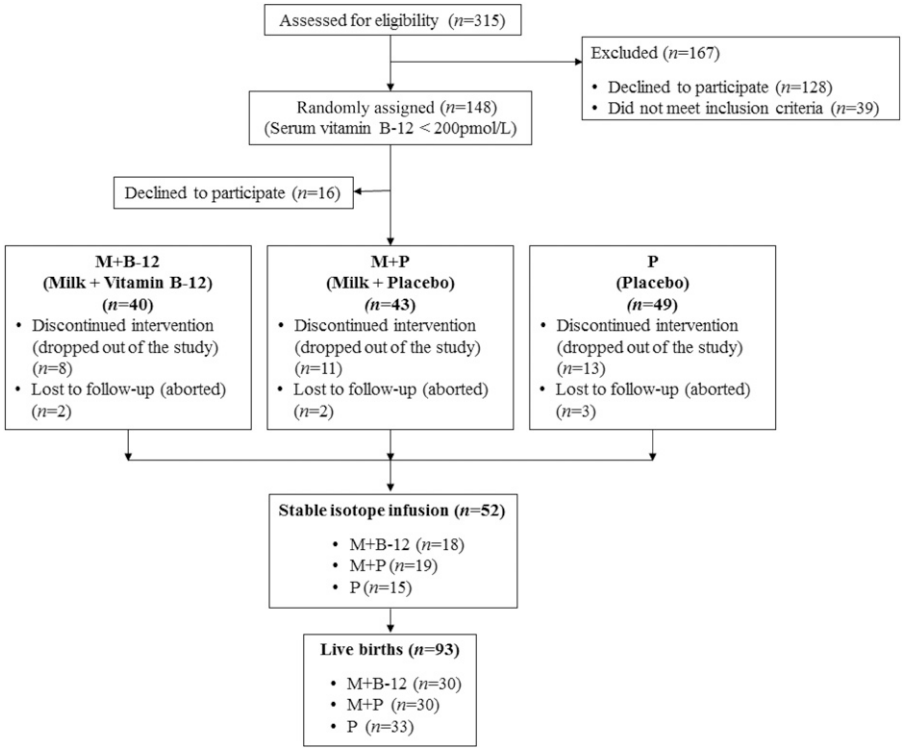

FIGURE 1 Flow chart of screening, recruitment, randomization, and follow-up of South Indian pregnant women who were randomly assigned to 1 of 3 groups (M+B-12, M+P, or P) and supplemented from recruitment (11 ± 2 wk of gestation) to delivery. M+B-12, milk and vitamin B-12; M+P, milk and placebo; P, placebo.

placenta were processed, sectioned, and examined after hematoxylin and eosin staining. The stained sections were read by a pathologist, and the findings were categorized as per recommendations of the Society for Pediatric Pathology (27) into 3 categories: uteroplacental insufficiency, fetoplacental insufficiency, and inflammatory lesions. Uteroplacental insufficiency included features of loss of integrity of maternal circulation

TABLE 1 Baseline characteristics of the South Indian pregnant women[1]

	Group			
Variables	M+B-12 (n = 30)	M+P (n = 30)	P (n = 33)	P^2
Demographic characteristics				
Age, y	23.7 ± 3.3	23.7 ± 2.8	22.7 ± 2.8	0.29
Parity, n (%)				
Primiparous	16 (53.3)	17 (56.7)	17 (51.5)	0.92
Education, n (%)				0.56
Up to high school	12 (40.0)	7 (23.3)	10 (30.3)	
Pre-university/diploma	11 (36.7)	14 (46.7)	11 (33.3)	
University and above	7 (23.3)	9 (30.0)	12 (36.4)	
Anthropometric measures at baseline				
Weight, kg	45.6 ± 5.1	45.2 ± 4.2	46.9 ± 6.8	0.46
Height, cm	154 ± 5.3	155 ± 9.8	154 ± 6.5	0.88
BMI, kg/m^2	19.1 ± 2.1	18.9 ± 2.5	19.7 ± 2.5	0.58
Blood pressure				
Systolic, mm Hg	112 ± 8	109 ± 8	109 ± 11	0.07
Diastolic, mm Hg	73 ± 6	69 ± 8	71 ± 9	0.06
Hemoglobin, g/dL	10.8 ± 1.4	10.9 ± 1.7	11.1 ± 1.5	0.79
Screening serum vitamin B-12, pmol/L	103 ± 38.6	107 ± 37.7	97 ± 34.8	0.59
Dietary intake at baseline				
Energy, kcal/d	1670 ± 368	1810 ± 533	1710 ± 426	0.48
Protein, % of energy	11.5 ± 1	11.6 ± 1	11.5 ± 1	0.37
Carbohydrate, % of energy	64.3 ± 4.3	65.2 ± 4.5	65.1 ± 4.3	0.76
Fat, % of energy	24.0 ± 3.8	26.3 ± 3.9	26.9 ± 3.8	0.41
Vitamin B-12, μg/d	1.6 ± 0.8	2.2 ± 1.6	1.5 ± 1.1	0.07
Methionine, g/d	0.9 ± 0.2	1.0 ± 0.3	0.9 ± 0.3	0.31
Folate, μg/d	240 ± 81.6	257 ± 98.9	248 ± 79.4	0.75

[1] Values are means ± SDs unless otherwise indicated. Subjects were randomly assigned to 1 of 3 groups (M+B-12, M+P, or P) and supplemented from recruitment (11 ± 2 wk of gestation) to delivery. M+B-12, milk + vitamin B-12; M+P, milk + placebo; P, placebo.
[2] Derived by using 1-factor ANOVA.

(retroplacental and intervillous hematomas) and changes in maternal vasculature (acute atherosis, persistent muscularization, mural hypertrophy) and villi (infarcts, syncytial knots, fibrin deposition, distal villous hypoplasia), leading to maternal underperfusion. Thrombotic lesions of fetal vessels (thrombi, intimal fibrin cushions, fibromuscular sclerosis) and villous changes (stromal vascular karyorrhexis, avascular villi) indicative of fetal thrombo-occlusive disease, along with chronic villitis with obliterative fetal vasculopathy, were grouped as fetoplacental insufficiency.

RNA and DNA extraction from placental samples. Formalin-fixed paraffin-embedded placental tissues from fetal sites of the placenta were used for gene expression and methylation analysis. Placental RNA and DNA were extracted from two 20-μm sections per specimen (28).

Real-time qPCR. Total RNA was reverse-transcribed and used for transcript abundance estimations. All qPCR reactions were performed in duplicate in a 10-μL reaction volume with a SYBR Green master mix. After exclusion of specimens with poorly preserved RNA, relative transcript abundance was calculated by using actin β (*ACTB*), β-2-microglobulin (*B2M*), and tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein ζ (*YWHAZ*) as control genes. Primer sequences for control and target genes are available in **Supplemental Table 1**. Relative expressions of the target genes [*MTR*, *VEGF*, fms-related tyrosine kinase 1 (*FLT1*), placental growth factor (*PGF*), *IGF1*, IGF 2 receptor (*IGF2R*), growth factor receptor-bound protein 10 (*GRB10*), pleckstrin homology like domain family A member 2 (*PHLDA2*), DNA methyltransferase (*DNMT*)1, and *DNMT3A*] were computed by modification of the ΔCt method (29).

DNA methylation, bisulfite modification, and MethyLight. CpGenome Universal Methylated DNA (Chemicon, Millipore) was used as 100% methylated control DNA. Unmethylated DNA was obtained from peripheral blood mononuclear cells by using established protocols (30). A total of 500 ng genomic DNA was bisulfite-modified by using the Zymo Research EZ DNA Methylation-Gold kit (Zymo Research) as per the manufacturer's instructions. MethyLight real-time PCR reactions were performed as described previously for the LINE-1–M1 region with the Alu region (ALU-C4) acting as the reference gene for normalization, and the percentage methylation ratio was calculated (31).

Methylation-sensitive high-resolution melt analysis. *VEGF* methylation levels were estimated by using primers that produced a 128-bp product. The methylation index of the samples based on the melt curve profile of the PCR products was derived according to Newman et al. (32).

Statistical analysis. A target sample size of 15 subjects/group was calculated to provide 80% power to detect a 10% difference (15% SD) of the kinetic parameters between the groups at a 5% level of significance (after Bonferroni adjustment for multiple comparisons) after accounting for a drop-out rate of ~20%. On the basis of an earlier study (33), for gene methylation in placental tissue, 10 subjects/group were required to detect a methylation difference of 5% with 80% power and a 5% level of significance. Because it was expected that high-quality placental tissue would be available in only approximately one-third of collected placentas, 30 women were recruited. Women were randomly assigned into the study by using a randomization list prepared by using permuted blocks of variable size.

Continuous data are presented as either means ± SDs or medians (quartile 1, quartile 3). All of the characteristics were compared between the intervention groups by either ANOVA or Kruskal-Wallis test on the basis of normality. Post hoc Tukey's multiple-comparison test was performed if data were normally distributed, and otherwise by the Mann-Whitney *U* test. The normality of distribution was examined by using Q-Q plots. Associations were examined using Spearman rank correlation coefficient. The changes from the first to third trimesters were examined by using paired *t* test or Wilcoxon Signed Rank test on the basis of the distribution of the variable. Combined vitamin B-12 was calculated according to the method suggested by Fedosov et al. (34). All of the analyses were performed by using the Statistical Package for Social Sciences version 18 (IBM SPSS Statistics for Windows; IBM Corp.). $P < 0.05$ was considered significant. Post hoc tests were performed and reported for all P values ≤0.06.

Results

Baseline characteristics. The study outline is shown in **Figure 1**. A total of 148 subjects with low vitamin B-12 status were randomly assigned into 1 of 3 parallel groups; M+B-12, $n = 40$; M+P, $n = 43$; and P, $n = 49$. As the study progressed, 39 subjects dropped out of the study. The enrollment and follow-up were from December 2010 to February 2014. The intervention was started at recruitment and was continued until delivery. A total

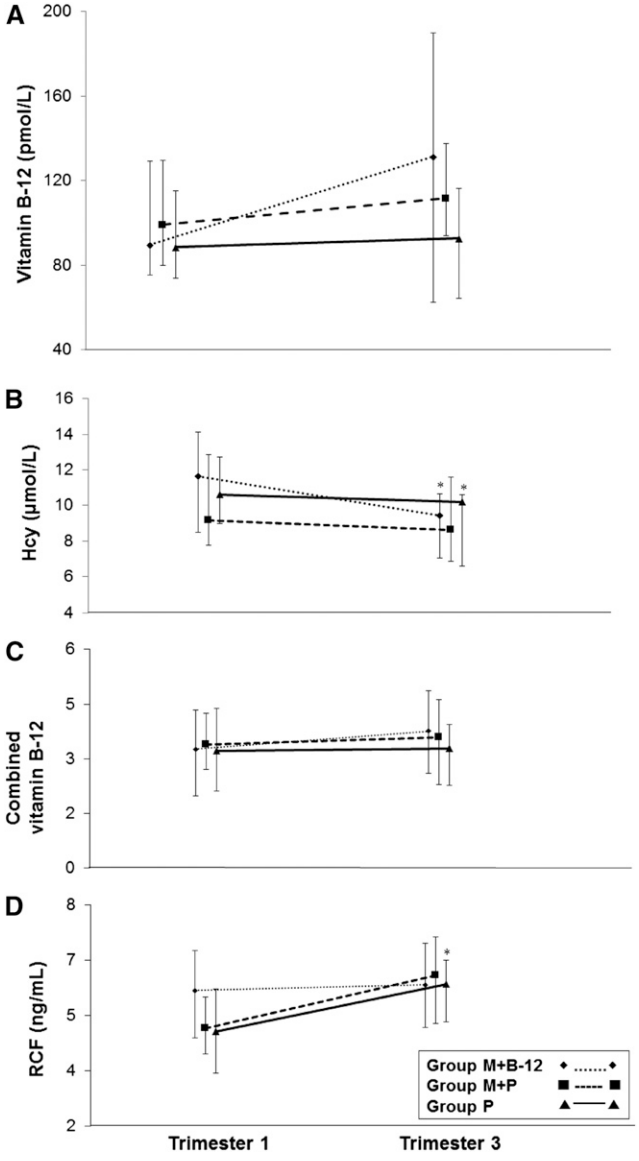

FIGURE 2 Effect of the intervention on maternal concentrations of serum vitamin B-12 (A), plasma homocysteine (B), combined vitamin B-12 (C), and RCF (D) in South Indian pregnant women who were randomly assigned to 1 of 3 groups (M+B-12, M+P, or P) and supplemented from recruitment (11 ± 2 wk of gestation) to delivery. Values are median and IQRs, $n = 30$ in the first trimester and 26 in third trimester. *$P < 0.05$ (paired *t* test between trimester 1 and trimester 3). Hcy, total homocysteine; M+B-12, milk and vitamin B-12; M+P, milk and placebo; P, placebo; RCF, RBC folate.

of 93 subjects completed the study (M+B-12, n = 30; M+P, n = 30; and P, n = 33). All first-trimester measurements were performed at 11 ± 2 wk of gestation and third-trimester measurements were performed at 30 ± 2 wk of gestation. A random subset from each group underwent tracer kinetic studies as shown in Figure 1; there was no difference between these women and the others who did not undergo the tracer studies in terms of demographic or anthropometric characteristics (data not shown). The baseline (first-trimester) maternal characteristics are shown in **Table 1**, where maternal anthropometric, socioeconomic, and habitual dietary characteristics were comparable between the 3 study groups.

Dietary intake, compliance, and biochemical status. Weight-specific dietary intakes and food-group data collected through multiple dietary recalls in the second and third trimester are summarized in **Supplemental Table 2**. Weight-specific protein intakes were comparable between the groups (P = 0.13), but the third-trimester intake of green-leafy vegetables (GLVs) was higher in the P group (median: 0.3 g; IQR: 0.12, 0.37 g) than in the M+B-12 group (median: 0.15 g; IQR: 0.07, 0.26 g) (P = 0.057).

The mean compliance rate for vitamin B-12 and placebo tablet consumption was 83%. The compliance rates were not significantly different between groups. The number of milk packets consumed in the M+B-12 and M+P groups were similar (~7 packets/wk, 500 mL/d). Responses of plasma vitamin B-12 to the intervention regimen are presented in **Figure 2**.

The maternal third-trimester median serum vitamin B-12 concentration was 131 pmol/L (IQR: 59, 196 pmol/L) in the M+B-12 group, whereas in the P group the vitamin B-12 concentration was 93 pmol/L (IQR: 62, 122 pmol/L), although this difference was not significant (P = 0.11). As pregnancy progressed, the increment in vitamin B-12 concentration from the first to third trimester was 33 pmol/L (95% CI: −4 to 71.7 pmol/L; P = 0.078) in the M+B-12 group. Similarly, combined vitamin B-12 also increased from the first to third trimester in the M+B-12 group (P = 0.077). The homocysteine concentration decreased significantly from the first to the third trimester in the M+B-12 and P groups (P = 0.040 and 0.032, respectively). However, the third-trimester homocysteine and RBC folate concentrations were similar between the groups (both P > 0.1).

Stable isotope kinetics. The tracer kinetics study was carried out in a subset of subjects (total of 52 subjects; n = 18, 19, and 15 in the M+B-12, M+P, and P groups, respectively) at 30 ± 1 wk of gestation. The remethylation rates and percentage of transmethylation remethylated to methionine approached significance for differences between the 3 groups (P = 0.057), such that the rates and percentages tended to be greater in the M+B-12 group than in the M+P group (P = 0.080 and P = 0.053, respectively) with neither the M+B-12 nor M+P groups differing from the P group (P > 0.1) (**Table 2**). The percentage of transmethylation transsulfurated to cysteine tended to be lower in the M+B-12 group than in the M+P group alone (P = 0.053) (Table 2). The phenylalanine

TABLE 2 Weight-specific methionine, glycine, serine, and phenylalanine kinetics measured in the third trimester in South Indian pregnant women[1]

	Group			
Kinetics, μmol · kg^{-1} · h^{-1}	M+B-12 (n = 18)	M+P (n = 19)	P (n = 15)	P^2
Methionine kinetics				
Total methionine flux	20.7 ± 4.7	19.0 ± 3.4	20.1 ± 4.6	0.51
Remethylation	5.1 ± 1.7[a]	4.1 ± 1.0[b]	5.0 ± 1.4[a,b]	0.057
Transsulfuration	5.2 ± 1.6	5.8 ± 1.8	6.4 ± 1.6	0.16
Transmethylation	10.4 ± 2.8	9.9 ± 2.1	11.5 ± 2.4	0.21
Transmethylation pathway				
Remethylated to methionine, %	49.5 ± 10.5[a]	42.3 ± 8.4[b]	44.2 ± 8.1[a,b]	0.057
Transsulfurated to cysteine, %	50.5 ± 10.5[a]	57.7 ± 8.4[b]	55.8 ± 8.1[a,b]	0.057
Glycine kinetics				
Total glycine flux	115 ± 19.5	111 ± 23.4	121 ± 16.7	0.35
Endogenous flux	107 ± 19.5	103 ± 23.4	113 ± 16.7	0.35
Flux derived from serine	7.3 ± 3.7	6.3 ± 2.8	7.3 ± 2.4	0.51
Serine kinetics				
Total serine flux	91.2 ± 18.3	94.2 ± 20.9	96.1 ± 17.3	0.77
Endogenous flux	85.2 ± 18.3	88.2 ± 20.9	90.1 ± 17.3	0.77
Flux derived from glycine	13.2 ± 3.3	12.5 ± 4.1	14.6 ± 4.1	0.35
Glycine flux to serine, %	11.4	11.3	11.9	0.56
Serine flux to glycine, %	8.0	6.7	7.6	0.70
Phenylalanine and tyrosine kinetics				
Total Phe flux	37.3 ± 4.2	34.9 ± 5.9	38.1 ± 6.5	0.22
Endogenous Phe flux	33.3 ± 4.2	30.9 ± 5.9	34.1 ± 6.5	0.22
Total Tyr flux	118 ± 34.2	119 ± 39.3	117 ± 23.5	0.99
Endogenous Tyr flux	117 ± 34.2	118 ± 39.3	116 ± 23.5	0.99
Phe hydroxylation to Tyr (index of protein oxidation)	11.2 ± 3.1	10.8 ± 3.0	12.1 ± 3.6	0.46
Phe not hydroxylated (index of protein synthesis)	26.0 ± 4.2	24.1 ± 4.8	26.4 ± 6.0	0.35

[1] Values are means ± SDs unless otherwise indicated. Subjects were randomly assigned to 1 of 3 groups (M+B-12, M+P, or P) and supplemented from recruitment (11 ± 2 wk of gestation) to delivery. Labeled means in a row without a common superscript letter differ by post hoc Tukey's test, $P \leq 0.06$. M+B-12, milk + vitamin B-12; M+P, milk + placebo; P, placebo.
[2] Derived by using 1-factor ANOVA.

kinetics (representative of whole-body protein breakdown rate) as well as glycine and serine kinetics were not different between the 3 groups. There were also no differences between the groups in the proportions of glycine flux derived from serine and serine flux derived from glycine (~7.0% and 11.5%, respectively).

Plasma amino acid profiles at the third trimester of pregnancy. Fasting plasma free amino acid concentrations assessed at the third trimester of pregnancy are presented in **Table 3**. Plasma concentrations of asparagine (P = 0.037), glycine (P = 0.004), alanine (P = 0.001), and ornithine (P = 0.055) were significantly different between the 3 groups. Although concentrations of asparagine were significantly higher in the P group than in the M+B-12 group (P = 0.038), concentrations of glycine, alanine, and ornithine were significantly higher in the P group than in the M+P group (P = 0.003, P = 0.001, and P = 0.051, respectively). The concentrations of the all other amino acids were comparable between the groups.

Delivery characteristics and newborn measurements. GWG from baseline to the second trimester was similar between the groups (**Table 4**). GWG from the second to the third trimester differed between the groups, such that it was higher in the M+B-12 group than in the P group (P = 0.027) (Table 4). The proportions of maternal morbidities, preterm births, and SGA births were comparable between the groups. Birth weights and gestational age at delivery were not significantly different between the 3 groups (all $P > 0.1$).

TABLE 3 Plasma amino acid concentrations (μmol/L) measured in the third trimester in South Indian pregnant women[1]

	Group			
Variables	M+B-12 (n = 18)	M+P (n = 19)	P (n = 15)	P^2
NH_3	16.0 ± 7.6	16.8 ± 6.2	14.9 ± 6.3	0.70
Histidine	78.8 ± 10.0	79.8 ± 12.5	85.6 ± 12.7	0.22
Asparagine	42.0 ± 7.6[a]	43.8 ± 9.5[a,b]	52.0 ± 16.1[b]	0.03
Serine	75.6 ± 13.7	72.4 ± 16.4	82.7 ± 15.5	0.14
Glutamine	390 ± 64.6	394 ± 51.9	416 ± 53.4	0.39
Arginine	47.9 ± 9.0	44.8 ± 10.1	53.1 ± 16.0	0.13
Glycine	174 ± 34.6[a,b]	153 ± 30.0[a]	191 ± 30.1[b]	<0.01
Aspartate	4.0 ± 2.0	3.3 ± 1.5	3.7 ± 0.82	0.33
Citrulline	20.4 ± 4.2	19.8 ± 4.5	19.9 ± 4.8	0.92
Glutamate	61.7 ± 11.3	58.9 ± 10.2	63.0 ± 16.0	0.61
Threonine	131 ± 26.6	132 ± 26.0	133 ± 35.0	0.98
Alanine	337 ± 60.3[a,b]	298 ± 53.0[a]	377 ± 54.6[b]	<0.01
Proline	150 ± 31.6	144 ± 32.3	164 ± 24.6	0.17
Ornithine	34.1 ± 7.9[a,b]	32.7 ± 6.4[a]	38.8 ± 7.6[b]	0.05
Cystine	15.8 ± 5.3	15.2 ± 5.0	15.3 ± 6.2	0.93
Lysine	97.8 ± 5.0	96.0 ± 17.6	96.3 ± 25.6	0.96
Tyrosine	51.5 ± 9.8	48.8 ± 8.3	53.0 ± 6.8	0.33
Methionine	16.9 ± 4.5	16.7 ± 3.0	18.7 ± 3.0	0.22
Valine	162 ± 20.3	157 ± 21.5	167 ± 30.6	0.46
Isoleucine	48.3 ± 8.0	44.8 ± 7.5	48.1 ± 8.8	0.35
Leucine	74.5 ± 13.0	70.9 ± 11.0	71.0 ± 15.5	0.66
Phenylalanine	51.4 ± 7.1	47.7 ± 5.8	51.1 ± 8.0	0.20
Tryptophan	28.9 ± 3.7	30.0 ± 5.2	28.6 ± 5.0	0.67

[1] Values are means ± SDs unless otherwise indicated. Subjects were randomly assigned to 1 of 3 groups (M+B-12, M+P, or P) and supplemented from recruitment (11 ± 2 wk of gestation) to delivery. Labeled means in a row without a common superscript letter differ by post hoc Tukey's test, $P \leq 0.06$. M+B-12, milk + vitamin B-12; M+P, milk + placebo; P, placebo.
[2] Derived by using 1-factor ANOVA.

Placental histopathologic and molecular assessment. Placentas were available from 66 births (**Supplemental Table 3**). The linear measurements of length, breadth, and trimmed placental weight were similar between the groups. Of the 66 placentas, 31 had well-preserved RNA that could be used for gene expression assays. The placental expression of the genes tested was similar between groups (**Supplemental Figure 1**); methylation of the *VEGF* promoter-associated region did not show any significant difference between the 3 groups. Quantitative LINE-1 methylation of placental samples was similar between the 3 groups (data not shown).

Discussion

This open-label, randomized controlled intervention trial conducted in pregnant South Indian women with low vitamin B-12 status found that balanced protein-energy and vitamin B-12 supplementation increased the remethylation rates of the methionine cycle. Although there was good compliance, there only was a trend toward an increased concentration of maternal serum vitamin B-12 in the third trimester in the M+B-12 group that was supplemented with milk and vitamin B-12, which is similar to the findings of a previous study during pregnancy and early lactation that showed that vitamin B-12 supplementation (50 μg/d compared with 10 μg/d in the present study) improved maternal plasma and breast-milk vitamin B-12 concentrations (16). To the best of our knowledge, this is the first trial that assessed the combinatorial effect of vitamin B-12 supplementation along with balanced energy protein on the basis of dietary source on amino acid kinetics in pregnant vitamin B-12–deficient Indian women.

The percentage of transmethylation to methionine was higher and transsulfuration to cysteine was lower (both approaching significance) in women who were supplemented with both milk and vitamin B-12 (M+B-12 group) but not in those who were supplemented with milk alone (M+P group) compared with the P group. The remethylation rate was lower in the M+P group than in the M+B-12 and P groups, such that the remethylation rate was lower in women who were supplemented with milk alone than in those who were supplemented with milk plus vitamin B-12 and with those who were not supplemented. This indicates that vitamin B-12, over and above balanced protein and energy supplementation, is critical for optimal functioning of the methionine cycle in the third trimester of pregnancy in Indian women with low vitamin B-12 status in early pregnancy. These findings are similar to those of a study conducted in well-nourished pregnant American women, in whom transmethylation rates increased from the first to the third trimester (6). The decrease in transsulfuration rate without any change in the remethylation rate with milk and vitamin B-12 supplementation points to a general conservation of methionine, possibly because of low maternal protein stores and the need to provide increased fetal needs. On the other hand, a relative deficiency of vitamin B-6, which is a cofactor necessary for transsulfuration, cannot be ruled out. For example, earlier studies in Indians have shown that vitamin B-6 deficiency is not unexpected and documented a prevalence of ~67% in affluent Indian children (35), which is likely to be similar in adults. The remethylation and transmethylation rates of the P group were not different from those of the M+B-12 group. A potential explanation for this observation could be the significantly higher intake of GLVs by the P group. GLVs are a well-known rich source of folate, and significantly depressed remethylation rates have been reported previously in

TABLE 4 Characteristics of the course of pregnancy, deliveries, and anthropometric measures of the neonates at birth[1]

Variables	Group			P^2
	M+B-12 (n = 29)	M+P (n = 29)	P (n = 31)	
Gestational weight gain, kg/wk				
Baseline to second trimester	0.49 ± 0.16	0.44 ± 0.16	0.53 ± 0.25	0.61
Second to third trimester	0.54 ± 0.18[a]	0.50 ± 0.23[a,b]	0.41 ± 0.22[b]	0.036
Maternal morbidity,[3] n (%)				—
Anemia	3 (11)	4 (17)	1 (4)	
GDM	5 (19)	3 (13)	3 (11)	
PROM	2 (7)	0	1 (4)	
PIH	0	2 (9)	0	
Birth outcomes,[3] n (%)				—
Preterm[4]	1 (3)	3 (10)	3 (10)	
SGA	7 (24)	5 (17)	6 (19)	
IUGR	4 (14)	0	1 (3)	
Sex, n (%)				
Male	14 (48)[a,b]	12 (41)[a]	22 (71)[b]	0.054
Female	15 (52)	17 (59)	9 (29)	—
Type of delivery, n (%)				
Normal	19 (63)	22 (73)	27 (82)	0.26
LSCD	11 (37)	8 (27)	6 (18)	—
Neonates				
Birth weight, g	2787 ± 357	2927 ± 482	2989 ± 405	0.17
Gestational age at birth, wk	38.1 ± 3.9	37.9 ± 3.1	38.0 ± 3.4	0.99
Length, cm	49.0 ± 2.1	49.4 ± 2.4	49.7 ± 2.1	0.59
MUAC, cm	9.2 ± 0.9	9.5 ± 1.1	9.4 ± 0.8	0.60
Head circumference, cm	32.9 ± 1.2	33.4 ± 1.4	33.5 ± 1.1	0.24
Chest circumference, cm	30.5 ± 1.6	31.1 ± 2.5	30.9 ± 1.7	0.58
Sum of skinfold thicknesses, cm	13.3 ± 2.4	13.8 ± 3.6	13.5 ± 2.5	0.78

[1] Values are means ± SDs unless otherwise indicated. Labeled means in a row without a common superscript letter differ by post hoc Tukey's test, $P \leq 0.06$. GDM, GDM, gestational diabetes mellitus; IUGR, intrauterine growth restriction; LSCD, lower segment cesarean delivery; M+B-12, milk + vitamin B-12; M+P, milk + placebo; MUAC, midupper arm circumference; P, placebo; PIH, pregnancy-induced hypertension; PROM, premature rupture of membranes; SGA, small for gestational age.
[2] Derived by using 1-factor ANOVA.
[3] Statistical test not done due to very small numbers within group.
[4] Preterm: gestational age at birth <37 wk.

folate-deficient human subjects, thereby supporting this line of reasoning (36).

Decreased concentrations of maternal plasma amino acids have been reported during pregnancy (37, 38) due to increased placental or fetus uptake for protein synthesis and oxidation (39). In light of pregnancy-associated hypoaminoacidemia in the fasting state, with specific reductions in glucogenic amino acids such as alanine, serine, threonine, glutamine, and glutamate that begin in early gestation and persist throughout pregnancy (37), maternal amino acid concentrations could be indicators of maternal protein intake and metabolism, which directly affect fetal growth. This is indicated by reports of positive associations between maternal plasma concentrations of total amino acids as well as of serine, lysine, proline, ornithine, and arginine with birth weight (40). In the present study, the plasma concentrations of 4 nonessential amino acids (i.e., asparagine, glycine, alanine, and ornithine) were significantly higher in the P group. Glycine is a critical amino acid during fetal growth, is required as a one-carbon donor, and is necessary for both the synthesis and methylation of DNA during fetal growth (41), as well as the higher fetal demand for collagen synthesis (42). It is also involved in the disposal of excess methionine through glycine-N-methyl transferase (43). The additional amount of methionine that was ingested in milk (500 mL/d) in the intervention groups (M+B-12 and M+P) was 415 mg/d (~9 mg · kg^{-1} · d^{-1}). Dimethyl glycine or sarcosine concentrations were not measured, and it is therefore not possible to partition the reason for the lower glycine concentrations between fetal growth requirements and disposal of excess methionine.

A possible limitation of this study could be that all of the study subjects, including those in the P group, were given nutritional counseling throughout pregnancy. This could have resulted in the comparable energy intakes across groups. Furthermore, the subjects were recruited in the study on the basis of their serum vitamin B-12 concentrations, although defining vitamin B-12 deficiency solely on the basis of serum vitamin B-12 concentrations is not ideal, as reviewed by Aparicio-Ugarriza et al. (44). Finally, the open-label nature of this trial is likely to have been a limitation. Although the researchers and subjects knew whether or not they were being supplemented with milk, the vitamin B-12 and the placebo tablets were unlabeled (coded) and neither the subjects nor the research assistants who supplied these tablets were aware of the composition of the tablets.

Many previous studies have reported differential placental and offspring DNA methylation of various loci in human intrauterine growth restriction and SGA placentas associated with maternal dietary differences, including the Dutch Hunger

Winter and rural Gambia–based studies (2–4, 45). In contrast to recent findings from rural Gambia (4), there was no influence of maternal dietary supplementation on either placental *VEGF* promoter region or LINE-1 methylation. There could be multiple reasons for this. First, although the present supplementation study assessed placental DNA methylation, a natural seasonal variation in maternal nutrition status in rural Gambia was used to understand associations between maternal nutritional intakes with the DNA methylation of specific metastable epialleles (MEs) in offspring peripheral blood lymphocyte DNA. Second, the methylation level of ME, by definition, can respond to maternal nutrition status only during the periconceptional period, because the high degree of similarity in methylation levels of the MEs in tissues derived from different germ layers point to a time window of methylation regulation before differentiation of the germ layers during the periconceptional period (46). Our supplementation regime was from the late first trimester until birth and it is possible that placental LINE-1 and *VEGF* promoter methylation might not respond to maternal protein or vitamin B-12 supplementation during that time window. Third, in the Gambian study, an inverse association was observed between maternal periconceptional homocysteine status and offspring ME methylation levels. In contrast, maternal first-trimester homocysteine status was not different between the study groups in the current study. Finally, the Gambian study found consistently higher ME methylation in offspring born during the protein-energy–limited, and therefore methyl donor–limited, rainy season. This argues against the dietary availability of methyl donors as a limiting factor for DNA methylation. It is likely that DNA methylation is a combined mark of several environmental influences, of which dietary methyl donors are only one part; several others exist, such as dietary energy density, smoking, alcohol, environmental toxins, and infectious agents, some of which are known to influence the offspring epigenome (47) in the intrauterine (maternal smoking) or postnatal (maternal nurturing) stage.

In summary, this is the first study to our knowledge to systematically evaluate the effect of vitamin B-12 supplementation in the maternal diet on methionine kinetics, placental gene expression, and DNA methylation in pregnant women. Maternal dietary supplementation with vitamin B-12 improved one-carbon cycle kinetics, particularly the decline in transsulfuration indicating conservation of methionine. This suggests that macronutrient supplementation during pregnancy, even though it is food based, needs to be accompanied by appropriate micronutrient supplementation for optimal effects. The supplementation regime did not affect either placental expression of genes in methionine pathways or placental LINE-1 methylation, which argues against the specific role of dietary supplementation of methyl donors to modify methylation patterns in the offspring. Future intervention studies in low-BMI women in the periconceptional state is needed to provide more information on the role of dietary factors on birth outcomes and offspring DNA methylation.

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