Low plasma vitamin B-12 is associated with a lower pregnancy-associated rise in plasma free choline in Canadian pregnant women and lower postnatal growth rates in their male infants\(^1\)\(^2\)\(^3\)

Brian TF Wu, Sheila M Innis, Kelly A Mulder, Roger A Dyer and D Janette King

ABSTRACT
Background: Choline needs are increased in pregnancy. Choline can be used as a source of methyl for homocysteine remethylation to methionine, but choline synthesis requires methyls from methionine. Vitamin B-12 deficiency increases choline use for homocysteine methylation.

Objectives: We investigated whether poor vitamin B-12 status occurs and contributes to low plasma choline and altered biomarkers of choline synthesis in pregnant women. With the use of a post hoc analysis, we addressed the association of maternal plasma vitamin B-12 status with postnatal growth rates in term infants.

Design: Blood was analyzed for a prospective study of 264 and 220 pregnant women at 16 and 36 wk of gestation, respectively, and 88 nonpregnant women as a reference.

Results: The proportion of women with a plasma total vitamin B-12 concentration <148 pmol/L (deficient) or 148–220 pmol/L (marginal) increased with pregnancy and pregnancy duration, which affected 3% and 9% of nonpregnant women, 10% and 21% of women at 16 wk of gestation, and 23% and 35% of women at 36 wk of gestation, respectively. Plasma free choline, betaine, and dimethylglycine were lower in women at 36 wk of gestation with a deficient or marginal compared with sufficient plasma total vitamin B-12 concentration (>220 pmol/L). Plasma total vitamin B-12 was positively associated with the increase in plasma free choline from midgestation to late gestation (\(P < 0.001\)). The postnatal growth rate to 9 mo was lower in infant boys of women classified as total vitamin B-12 deficient compared with sufficient.

Conclusion: This study shows that maternal vitamin B-12 status is related to choline status in late gestation in a folate-replete population and may be a determinant of infant growth even in the absence of undernutrition. Am J Clin Nutr doi: 10.3945/ajcn.113.060269.

INTRODUCTION

Recent years have seen a growing appreciation that choline requirements and metabolism are closely interrelated with methylation in the methionine-homocysteine cycle (1, 2). Choline has multiple, divergent roles, which include a component of phosphatidylcholine, in the neurotransmitter acetylcholine, and as a precursor for betaine. The role of betaine as a source of methyl for remethylation of homocysteine to methionine links choline to the methionine-homocysteine cycle (1, 2) (Figure 1).

The remethylation of homocysteine to methionine by betaine homocysteine methyltransferase (BHMT)\(^4\) functions as a parallel, alternate pathway to remethylation by the vitamin B-12–folate–dependent pathway (1, 2). Deficiency of vitamin B-12 or folate results in increased activity of the choline-betaine pathway for remethylation of homocysteine in humans and animals (3–5). However, endogenous choline synthesis relies on the transfer of methyl from methionine via S-adenosylmethionine to phosphatidylcholine that also generates S-adenosylhomocysteine, which is the immediate precursor of homocysteine (1, 2). As such, increased demands for choline synthesis may disturb the methionine-homocysteine cycle, because 3 molecules of methionine are used, and 3 homocysteine molecules are formed, for every choline synthesized (Figure 1).

Interest in choline during gestation has gained momentum because of the high demand for maternal choline to support the needs of the placenta and fetus and experimental evidence that choline deficiency during gestation alters fetal neural progenitor cell differentiation, migration apoptosis, and DNA methylation with long-term effects on learning (6–10). Evidence of a positive association between maternal choline and betaine in early gestation and cognitive development in infants at 18 mo of age was reported in one study (11), although another study showed no association between maternal serum choline in gestation and child intelligence quotient at 5 y of age (12). The increased demand for choline during pregnancy (2, 9, 10, 13) has raised concern that nutritional or other variables that limit the vitamin B-12–folate remethylation pathway may decrease maternal choline, with potential deleterious effects on offspring development.

The United States and Canada introduced the mandatory fortification of cereals with folic acid in 1998 as a public health strategy to reduce the incidence of pregnancies affected with neural tube defects. This program has been estimated to have reduced neural tube defect–affected pregnancies by \(\sim 50\%\) and has lowered plasma homocysteine and increased folate status.

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\(^2\) Supported by a grant from the Canadian Institutes of Health Research.

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\(^4\) Abbreviations used: BHMT, betaine-homocysteine methyltransferase; CDP, cytidine diphosphate; LC-MS/MS, liquid chromatography–tandem mass spectrometry; RBC, red blood cell.

Received February 10, 2013. Accepted for publication August 2, 2013. doi: 10.3945/ajcn.113.060269.
FIGURE 1. Simplified schematic showing dual roles of choline as the precursor of betaine and phosphatidylcholine but the need for methyl groups for endogenous choline synthesis. Enzymes shown are as follows: 1, betaine-homocysteine methyltransferase; 2, methionine synthase; 3, methionine adenosyltransferase; 4, S-adenosylhomocysteine hydrolase; 5, phosphatidylethanolamine N-methyltransferase; and 6, cystathionine β-synthase. CTP, cytidine triphosphate; Cys, cysteine; DAG, diacylglycerol; DMG, dimethylglycine; Hcy, homocysteine; MeB12, methyl vitamin B-12; Met, methionine; MeTHF, methyltetrahydrofolate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

(14). More recently, population surveys in Canada, which does not include vitamin B-12 in mandatory food fortification, have provided evidence of vitamin B-12 insufficiency (15, 16). Our objectives were to investigate whether poor vitamin B-12 status occurs and contributes to lower choline in midgestation and late gestation, healthy, pregnant women, and if so, whether this is likely to be explained by decreased endogenous phosphatidylcholine synthesis or increased use of choline-derived methyl for homocysteine remethylation. Because of evidence that poor maternal vitamin B-12 status in the absence of folate deficiency is associated with metabolic programming in children (17), post hoc analyses were used to explore altered early weight gains in infants born to mothers classified with or without plasma total vitamin B-12 deficiency (total vitamin B-12 concentration <148 pmol/L).

SUBJECTS AND METHODS

Subjects

Study participants were drawn from a prospective study of pregnant women and a cross-sectional study of nonpregnant women with young children in Vancouver, Canada. The prospective study was designed to evaluate the relation between dietary intake and blood status of n–3 fatty acids during pregnancy and child development (18–20). Nonpregnant reference women are a random subset of the first 88 women enrolled in an ongoing study on the covariance of n–3 fatty acids and associated nutrients between mothers and their children and their relation to child development. Fasting venous blood was collected at 16 wk gestation from n = 270 women and again from n = 220 of these women at 36 wk of gestation. Dietary intake was recorded for pregnant women at 16 and 36 wk of gestation by using a standardized food-frequency questionnaire (19) but not collected for the reference group of nonpregnant women. Nutrient intakes from foods and beverages were estimated for vitamin B-12 (version 10.5; ESHA Research). Total choline intakes were estimated by using the USDA database on choline in foods (Release Two) (21). Data on infant birth weight were collected from hospital records, and infant growth was measured and feeding recorded by the research study staff at postnatal study follow-up visits. For the purpose of this study, an infant was considered breastfed as long as the intake of a human-milk substitute or bottle feeds with cow milk did not exceed 250 mL/wk. The protocol was approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia and the British Columbia’s Children’s and Women’s Hospital. All participants provided written informed consent before participation. Subjects in this study were enrolled in studies registered at clinicaltrials.gov as NCT01112930 and NCT00620672.

Sample collection and biochemical assessment

Venous blood was collected into tubes containing EDTA as the anticoagulant. Immediately on collection, blood samples were centrifuged at 2000 × g at 4°C for 15 min, and the plasma was recovered, divided into aliquots, and frozen at −70°C. Red blood cells (RBCs) were washed 3 times with saline, divided into aliquots, and frozen at −70°C. Plasma free choline, betaine, and dimethylglycine were quantified by using normal phase liquid chromatography–tandem mass spectrometry (LC-MS/MS) with stable-isotope–labeled internal standards, as previously described (22). Plasma methionine, homocysteine, and cysteine were also quantified by using LC-MS/MS with deuterium-labeled methionine, homocysteine (d4-methionine, D-3262; d8-homocysteine, D-3030; C/D/N Isotopes), and cysteine (d3-cysteine; DLM-899 Cambridge Isotopes) as internal standards. The LC-MS/MS was a Waters ACQUITY UPLC system connected to a Quatro micro triple quadrupole mass spectrometer (Waters Canada). Briefly, deuterium-labeled methionine, homocysteine, and cysteine were added to 50 µL plasma, dithiothreitol was added at a concentration of 62.5 mmol/L, and the plasma was incubated for 15 min at room temperature. Proteins were precipitated with 1 vol acetonitrile, and the supernatant fluid was recovered after centrifugation at 14,000 × g for 10 min. Chromatography was accomplished on a C18, 2.1 mm × 50 mm column with a C8 guard column (Agilent Technologies) with a binary mobile phase gradient in which the first phase was water. The second phase was methanol and was increased from 10% to 100% with 0.2% heptfluorobutric acid in each phase as an ion-pairing agent. The sample injection volume was 4 µL and was accomplished with an autosampler and temperature-controlled sample chamber held at 5°C. Plasma folate was measured by using an ion-capture assay, and total vitamin B-12 was quantified by using a microparticle enzyme immunoassay with an AxSym Analyzer (Abbott Laboratories) as per the manufacturer’s instructions. Intersay and intraassay CVs were as follows: for choline, 3.8% and 2.5%, respectively; for betaine, 3.5% and 2.2%, respectively; for dimethylglycine, 3.8% and 2.4%, respectively; for methionine, 2% and 1%, respectively; for homocysteine, 1.7% and 2.3%, respectively; for cysteine, 1.4% and 1.2%, respectively; for folate, 3.5% and 2.9%, respectively; and for total vitamin B-12, 3.2% and 1.7%, respectively.

Phosphatidylcholine in RBCs, the brain, and other organs is characterized by higher palmitic (16:0) and linoleic acid (18:2n–6) but lower arachidonic acid (20:4n–6) and docosahexaenoic acid (22:6n–3) than phosphatidylethanolamine (18, 23, 24). Recent studies have shown that the major phosphatidylcholine species

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synthesized from cytidine diphosphate (CDP) choline and diglyceride are 16:0/18:1, 18:0/18:2, and 18:1/18:1, whereas phosphatidylcholine synthesized by methylation of phosphatidylethanolamine has higher unsaturated fatty acids (18:1/18:1, 18:0/18:2, 18:2/20:4, 18:1/20:4, 18:0/20:4, and 18:0/22:6) (25). Therefore, we used an analysis of RBC phosphatidylcholine fatty acids at 36 wk gestation for the subset of 107 women who took no supplemental n−3 fatty acids during their pregnancy as biomarkers of altered phosphatidylcholine synthesis involving methyl transfer from methionine. RBC total lipids were extracted, phosphatidylcholine was separated, and its fatty acids were determined by using routine capillary column gas liquid chromatography (26).

Data analysis
The normality of data was determined by using the Kolmogorov-Smirnov test, and data are presented as means ± SDs and medians and 25th–75th IQRs. Plasma concentrations of variables of interest were compared between groups by using the Mann-Whitney U test or 1-factor ANOVA with a Tukey’s honestly significant difference post hoc test. Spearman’s ρ correlation analysis and Pearson’s correlation analysis were used to assess significant associations in choline, betaine, dimethylglycine, folate, total vitamin B-12, methionine, homocysteine, and cysteine. Pearson’s correlation analysis was used to determine the relation between the absolute (µmol/L) and percentage of change in plasma free choline from 16 to 36 wk of gestation and the ln-transformed plasma total vitamin B-12. Cutoffs for the classification of plasma total vitamin B-12 deficiency and marginal and sufficient status were <148, 148–220, and >220 pmol/L on the basis of MacFarlane et al (15) and Miller et al (27). Multiple regression analyses were used to explore potential differences in the postnatal growth rate, which was calculated as grams of weight gain per kilograms of birth weight per day of boys and girls born to mothers with a plasma total vitamin B-12 concentration <148 compared with ≥148 pmol/L at 36 wk of gestation, with breastfeeding controlled for. Because of the exploratory nature of these analyses, additional factors that might have influenced postnatal growth were not included. All statistical analyses were done with SPSS 20.0 software (SPSS Inc), and statistical significance was defined as P < 0.05 unless otherwise stated.

RESULTS

Subject characteristics
The mean ± SD (range) age of pregnant women was 33.1 ± 3.84 y (20–41 y), with 41% of the nonpregnant women between 20–39 y of age and 59% of women between 40–49 y of age. Of pregnant and nonpregnant women, 74% and 65% of women, respectively, reported that they had attended university, and 72% and 67% of women, respectively, were of white background. Women of Asian background made up 15% and 22% of pregnant and nonpregnant women, respectively. All pregnant women reported that they took a prenatal multivitamin supplement, although data on compliance were not noncollected. Only 6 women reported that they smoked at any time during their pregnancy. Insufficient blood was available for analyses of 6 of 270 pregnant women at 16 wk of gestation, and 220 women also gave blood at 36 wk of gestation. However, of the women seen at 36 wk of gestation, 1 infant was born with each of a congenital neurological malformation, metabolic disorder, and intrauterine growth retardation, and 217 women delivered a healthy term infant. Preterm delivery, miscarriage, pregnancy termination, or preeclampsia occurred in 13 women; 37 women were lost to follow-up before 36 wk of gestation, and data on birth weight were not retrieved for 1 infant.

The distribution of plasma free choline, betaine, and dimethylglycine as well as methionine, homocysteine, cysteine, folate, and total vitamin B-12 were skewed at 16 and 36 wk of gestation (n = 264 and 220, respectively; P < 0.05) but not in the reference group of nonpregnant women (n = 88; P > 0.05) (Table 1). Plasma free choline increased significantly from 16 to 36 wk of gestation (P < 0.05) to reach concentrations significantly higher than those of nonpregnant women (P < 0.05), and this result was not explained by an increase in the estimated dietary total choline intake (mean ± SD: 391 ± 101 and 401 ± 107 mg/d at 16 and 36 wk of gestation, respectively; P > 0.05). In contrast to free choline, plasma betaine concentrations were >50% lower in nonpregnant women.

| TABLE 1 |
| Plasma choline, methionine, and related metabolites, folate, and vitamin B-12 in pregnant women and a reference group of nonpregnant women

<table>
<thead>
<tr>
<th></th>
<th>16 wk of gestation (n = 264)</th>
<th>36 wk of gestation (n = 220)</th>
<th>Nonpregnant women (n = 88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline (µmol/L)</td>
<td>6.90 (5.96–8.10)</td>
<td>7.21 ± 1.84</td>
<td>9.40 (8.30–11.3)</td>
</tr>
<tr>
<td>Betaine (µmol/L)</td>
<td>13.0 (10.6–15.8)</td>
<td>13.7 ± 4.22</td>
<td>13.1 (11.3–14.8)</td>
</tr>
<tr>
<td>Dimethylglycine (µmol/L)</td>
<td>1.00 (0.80–1.40)</td>
<td>1.12 ± 0.48</td>
<td>1.30 (1.05–1.70)</td>
</tr>
<tr>
<td>Methionine (µmol/L)</td>
<td>20.6 (18.3–23.9)</td>
<td>21.3 ± 4.87</td>
<td>22.1 (19.4–25.4)</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>4.10 (3.50–4.80)</td>
<td>4.26 ± 1.37</td>
<td>4.80 (4.20–5.70)</td>
</tr>
<tr>
<td>Cysteine (µmol/L)</td>
<td>206 (189-225); 205 ± 31.6</td>
<td>206 (191-228); 210 ± 29.1</td>
<td>330 (294-358); 325 ± 48.5</td>
</tr>
<tr>
<td>Folate (nmol/L)</td>
<td>35.9 (33.3–38.1)</td>
<td>35.5 ± 4.89</td>
<td>35.8 (32.8–38.8)</td>
</tr>
<tr>
<td>Total vitamin B-12 (pmol/L)</td>
<td>259 (199–351); 287 ± 126</td>
<td>206 (150–284)</td>
<td>224 ± 96.2</td>
</tr>
</tbody>
</table>

1 Data are medians (25th–75th percentiles); means ± SDs. Data for pregnant and nonpregnant women were skewed (P < 0.05) and normally distributed (P > 0.05), respectively, by using the Kolmogorov-Smirnov test.
2 Significantly different from nonpregnant women, P < 0.05 (Mann-Whitney U test).
3 Significantly different from gestational week 16, P < 0.05 (Mann-Whitney U test).
4 There were 0 and 1 woman at 16 and 36 wk of gestation, respectively, and 3 nonpregnant women with a plasma homocysteine concentration >13 µmol/L.
5 With the use of a plasma folate concentration <6.8 nmol/L, no subject was folate deficient.
6 With the use of a plasma total vitamin B-12 concentration <148 pmol/L, 27 and 51 women at 16 and 36 wk of gestation, respectively, and 3 nonpregnant women were total vitamin B-12 deficient.
pregnant than nonpregnant women \( (P < 0.001) \) and not different between women in midgestation and late gestation. The plasma dimethylglycine concentration was also lower in pregnant than nonpregnant women but was higher at 36 compared with 16 wk of gestation \( (P < 0.05) \). Plasma methionine, homocysteine, cysteine, and total vitamin B-12 concentrations were also significantly lower in pregnant women than in the reference group of nonpregnant women \( (P < 0.05) \), whereas plasma folate concentrations were higher \( (P < 0.05); \) Table 1). Plasma total vitamin B-12 was not different between nonpregnant women who were between 20 and 39 and between 40 and 49 y of age \( (408 \pm 166 \) and \( 417 \pm 152 \) mg/d, respectively; \( n = 36 \) and \( n = 52 \), respectively; \( P = 0.808 \)), and any age-related decrease in plasma total vitamin B-12 would underestimate, not overestimate, the extent of lowering of plasma total vitamin B-12 in pregnant women. Results for the subset of 217 women at 16 and 36 wk gestation who delivered healthy term infants were not different (see Supplemental Table 1 under “Supplemental data” in the online issue). See Supplemental Table 2 under “Supplemental data” in the online issue for data for plasma choline, methionine, related metabolites, and folate for the reference women grouped by age. With the use of a deficiency cutoff of \(< 6.8 \) nmol/L for plasma folate, no woman in the study was folate deficient. None of the women at 16 wk of gestation, 1 of 220 women at 36 wk of gestation, and 3 of 88 nonpregnant women had a plasma homocysteine concentrations \( > 13 \) µmol/L. With the use of cutoffs of \(< 148, 148–220, \) or \(> 220 \) pmol/L for deficient, marginal, or sufficient plasma total vitamin B-12, respectively \( (15, 27), 27 \) women \( (10\%) \) and \( 55 \) women \( (21\%) \) at 16 wk of gestation, and \( 51 \) women \( (23\%) \) and \( 77 \) women \( (35\%) \) at 36 wk of gestation met the criteria of possible total vitamin B-12 deficiency or marginal status, respectively (Table 1). Dietary vitamin B-12 intake was significantly lower in pregnant women with a plasma total vitamin B-12 concentration <148 pmol/L than in women with a total vitamin B-12 concentration \( > 220 \) pmol/L \( (\text{mean} \pm \text{SD}: 5.16 \pm 1.95 \) and \( 6.37 \pm 2.14 \) pmol/L, respectively; \( n = 51 \) and \( n = 92 \), respectively; \( P < 0.01 \)), with intermediate vitamin B-12 intakes in pregnant women with a plasma total vitamin B-12 concentration of 148–220 pmol/L \( (5.82 \pm 1.95 \) pmol/L; \( n = 77 \)). In contrast, only 3 nonpregnant women \( (3\%) \) and 8 nonpregnant women \( (9\%) \) met the criteria of total vitamin B-12 deficiency or marginal status, respectively.

**Associations between plasma free choline, methyl nutrients, and vitamin B-12**

Plasma free choline was significantly, positively associated with plasma betaine and dimethylglycine in both pregnant and nonpregnant women (Table 2). Results for 217 women seen at 16 and 36 wk of gestation and who delivered a healthy term infant were not different (see Supplemental Table 3 under “Supplemental data” in the online issue). Plasma free choline, betaine, and dimethylglycine were all positively associated with plasma methionine and cysteine in pregnant women at 36 wk \( (P < 0.01) \). In contrast to late pregnancy, plasma free choline, betaine, and dimethylglycine showed no significant association with cysteine in the reference group of nonpregnant women \( (P > 0.05) \), with a weaker relation between cysteine and free choline \( (P < 0.05) \) and no relation between betaine or dimethylglycine and cysteine at 16 wk of gestation (Table 2). No significant relations were shown between plasma free choline, betaine, or dimethylglycine and homocysteine (Table 2; see Supplemental Table 3 under “Supplemental data” in the online issue).

In contrast to choline, plasma homocysteine concentrations were inversely correlated with the plasma total vitamin B-12 at 16 and 36 wk of gestation \( (\rho = -0.153, P = 0.014; \rho = -0.208; P = 0.002, \) Table 2).

**TABLE 2**

<table>
<thead>
<tr>
<th>Choline</th>
<th>Methionine</th>
<th>Homocysteine</th>
<th>Cysteine</th>
<th>Betaine</th>
<th>Dimethylglycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational week 16 (( \rho ))</td>
<td>0.24 ( ^{2} )</td>
<td>0.11</td>
<td>0.14 ( ^{4} )</td>
<td>0.60 ( ^{2} )</td>
<td>0.42 ( ^{2} )</td>
</tr>
<tr>
<td>Gestational week 36 (( \rho ))</td>
<td>0.21 ( ^{2} )</td>
<td>0.07</td>
<td>0.25 ( ^{2} )</td>
<td>0.42 ( ^{2} )</td>
<td>0.43 ( ^{2} )</td>
</tr>
<tr>
<td>Nonpregnant (( \rho ))</td>
<td>0.33 ( ^{2} )</td>
<td>-0.01</td>
<td>0.08</td>
<td>0.30 ( ^{2} )</td>
<td>0.30 ( ^{2} )</td>
</tr>
<tr>
<td>Betaine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational week 16 (( \rho ))</td>
<td>0.20 ( ^{2} )</td>
<td>-0.09</td>
<td>0.07</td>
<td>-</td>
<td>0.52 ( ^{2} )</td>
</tr>
<tr>
<td>Gestational week 36 (( \rho ))</td>
<td>0.25 ( ^{2} )</td>
<td>0.01</td>
<td>0.20 ( ^{2} )</td>
<td>-</td>
<td>0.50 ( ^{2} )</td>
</tr>
<tr>
<td>Nonpregnant (( \rho ))</td>
<td>0.14</td>
<td>-0.04</td>
<td>0.05</td>
<td>-</td>
<td>0.49 ( ^{2} )</td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational week 16 (( \rho ))</td>
<td>0.24 ( ^{2} )</td>
<td>0.03</td>
<td>0.08</td>
<td>0.52 ( ^{2} )</td>
<td>-</td>
</tr>
<tr>
<td>Gestational week 36 (( \rho ))</td>
<td>0.18 ( ^{2} )</td>
<td>0.14 ( ^{4} )</td>
<td>0.21 ( ^{2} )</td>
<td>0.50 ( ^{2} )</td>
<td>-</td>
</tr>
<tr>
<td>Nonpregnant (( \rho ))</td>
<td>0.16</td>
<td>-0.12</td>
<td>0.05</td>
<td>0.49 ( ^{2} )</td>
<td>-</td>
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<tr>
<td>Homocysteine</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gestational week 16 (( \rho ))</td>
<td>0.19 ( ^{2} )</td>
<td>-</td>
<td>0.56 ( ^{2} )</td>
<td>-0.09</td>
<td>0.03</td>
</tr>
<tr>
<td>Gestational week 36 (( \rho ))</td>
<td>0.21 ( ^{2} )</td>
<td>-</td>
<td>0.53 ( ^{2} )</td>
<td>0.01</td>
<td>0.14 ( ^{4} )</td>
</tr>
<tr>
<td>Nonpregnant (( \rho ))</td>
<td>0.28 ( ^{2} )</td>
<td>-</td>
<td>0.69 ( ^{2} )</td>
<td>-0.04</td>
<td>-0.12</td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational week 16 (( \rho ))</td>
<td>0.38 ( ^{2} )</td>
<td>0.56 ( ^{2} )</td>
<td>-</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>Gestational week 36 (( \rho ))</td>
<td>0.36 ( ^{2} )</td>
<td>0.53 ( ^{2} )</td>
<td>-</td>
<td>0.20 ( ^{4} )</td>
<td>0.21 ( ^{4} )</td>
</tr>
<tr>
<td>Nonpregnant (( \rho ))</td>
<td>0.21 ( ^{4} )</td>
<td>0.09 ( ^{2} )</td>
<td>-</td>
<td>0.08</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\( ^{1} \) Data are Spearman’s \( \rho \) correlation for 264 and 220 women at 16 wk and 36 wk of gestation, respectively, and Pearson correlation analysis for 88 nonpregnant women.

\( ^{2} \) Significant association between indicated variables: \( ^{2} P < 0.01; ^{4} P < 0.05. \)
respectively) and plasma total vitamin B-12 in the reference group of nonpregnant women \((r = -0.267, P = 0.012)\). No significant associations were shown between plasma folate and homocysteine in midgestation or late gestation \((P > 0.05)\), but plasma folate was inversely related to plasma homocysteine in non-pregnant women \((P = 0.003)\). Notably, the plasma total vitamin B-12 showed consistent, positive associations with the plasma methionine:homocysteine ratio at 16 wk \((r = 0.190, P = 0.002)\) and 36 wk of gestation \((r = 0.201, P = 0.003)\) (Figure 2). A significant positive association was also present between plasma total vitamin B-12 and the methionine:homocysteine ratio in nonpregnant women \((r = 0.226, P = 0.034)\). There was no evidence of any significant association, either positive or negative, between plasma total vitamin B-12 and plasma free choline: betaine and betaine:dimethylglycine ratios in either pregnant or nonpregnant women \((P > 0.05; \text{data not shown})\).

Effect of vitamin B-12 status on plasma free choline

Midgestation and late-gestation women with deficient \(<148 \text{ pmol/L}\) or marginal \((148–220 \text{ pmol/L})\) plasma total vitamin B-12 has significantly lower plasma free choline, betaine, and dimethylglycine and higher plasma homocysteine compared with women at the same stage of gestation with a plasma total vitamin B-12 concentration \(>220 \text{ pmol/L}\) \((P < 0.05; \text{Table 3})\). In addition, the plasma total vitamin B-12 was positively associated with the absolute change \((\mu\text{mol/L})\) and relative changed (percentage of change) in plasma free choline from midgestation to late gestation by using paired data from 220 or 217 women who delivered healthy single birth infants \((P < 0.001)\) (Figure 3). Plasma choline:betaine and betaine:dimethylglycine ratios, as indexes of choline- or betaine-derived methyl utilization for homocysteine methylation, were not different in pregnant women with plasma total vitamin B-12 deficiency or insufficiency compared with sufficiency \((P > 0.05; \text{Table 3})\).

The pathway of phosphatidylcholine synthesis, from CDP choline and diglyceride, or by the sequential methylation of phosphatidylethanolamine, can influence the fatty acid composition of the phosphatidylcholine formed \((25)\), but dietary supplementation with 22:6n–3 increases RBC phosphatidylcholine 22:6n–3 and can decrease arachidonic acid \((20:4n–6)(18, 28, 29)\). The RBC phosphatidylcholine showed no evidence of significant differences in any major fatty acid \((16:0, 18:0, 18:1n–9, 18:2n–6, 18:3n–3, 20:4n–6, \text{or} 22:6n–3)\) in women at 36 wk of gestation who took no supplemental n–3 fatty acids when grouped as plasma total vitamin B-12 deficient, marginal, or sufficient \((n = 107, P > 0.05; \text{see Supplemental Table 4 under “Supplemental data” in the online issue})\).

Maternal vitamin B-12 status and infant postnatal growth

After adjustment for breastfeeding, boys born to mothers who were classified as plasma total vitamin B-12 deficient \(<148 \text{ pmol/L}\) at 36 wk of gestation grew more slowly from birth to 2, 6, and 9 mo of age than did boys born to mothers with a plasma total vitamin B-12 concentration \(\geq 148 \text{ pmol/L}\) \((\text{Table 4})\). Maternal plasma total vitamin B-12 status in gestation was not associated with any apparent difference in infant birth weight or with postnatal growth from birth to 9 mo of age in infant girls.

DISCUSSION

The current study was conducted in Canada after more than a decade of fortification of the food supply with folic acid, improved folate status, and a decrease in plasma homocysteine in the population but emerging evidence of vitamin B-12 inadequacy.
(14–16). Dietary deficiencies that decrease homocysteine remethylation via the vitamin B-12–folate–dependent pathway increase homocysteine remethylation by using methyl from betaine (1–5). However, the transfer of methyl from methionine to phosphatidylethanolamine is required for choline synthesis (Figure 1), and choline synthesis is increased in pregnancy, to phosphatidylethanolamine is required for choline synthesis betaine (1–5). However, the transfer of methyl from methionine increase homocysteine remethylation by using methyl from methylation via the vitamin B-12–folate–dependent pathway (14–16). Dietary deficiencies that decrease homocysteine remethylation.

Our results showed that plasma total vitamin B-12, the increase in free choline from 16 to 36 wk of gestation, and the plasma methionine:homocysteine ratio are provocative evidence that methyl nutrition affects methionine-homocysteine functioning in pregnancy. Associations between vitamin B-12 and choline in foods could offer an alternate explanation; however, this reasoning seems unlikely because of 36% increase in plasma free choline between 16 and 36 wk of gestation and lack of significant association between choline intake and plasma free choline at 36 wk of gestation.

Homocysteine can be metabolized by the 4 enzymes methionine synthase, BHMT, cystathionine β-synthase, and S-adenosylhomocysteine hydrolase (Figure 1). S-adenosylhomocysteine hydrolase is readily reversible but usually favors homocysteine synthesis. Methionine synthase and BHMT both catalyze the remethylation of homocysteine to methionine, whereas cystathionine β-synthase catalyzes the irreversible entry of homocysteine to the transsulfuration pathway leading to cysteine. Our results that combined analyses of methionine, homocysteine, cysteine, and choline and its metabolites highlighted the marked changes in methyl metabolism in gestation. Thus, although plasma free choline increased from midgestation to late gestation to reach concentrations greater than nonpregnant reference values, methionine, homocysteine, cysteine, betaine, and dimethylglycine all decreased by ≥35% compared with in nonpregnant women.

### Table 3

Plasma choline and metabolites in pregnant women grouped by plasma total vitamin B-12 status.

<table>
<thead>
<tr>
<th>Plasma total vitamin B-12</th>
<th>Deficient (&lt;148 pmol/L)</th>
<th>Marginal (148–220 pmol/L)</th>
<th>Sufficient (&gt;220 pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 wk of gestation (n)</td>
<td>51</td>
<td>77</td>
<td>92</td>
</tr>
<tr>
<td>Choline (μmol/L)²</td>
<td>9.01 ± 2.02²</td>
<td>9.49 ± 1.80²</td>
<td>10.6 ± 2.26</td>
</tr>
<tr>
<td>Betaine (μmol/L)²</td>
<td>12.8 ± 2.80³</td>
<td>13.0 ± 2.66³</td>
<td>14.1 ± 2.99</td>
</tr>
<tr>
<td>Dimethylglycine (μmol/L)²</td>
<td>1.39 ± 0.50</td>
<td>1.30 ± 0.59³</td>
<td>1.52 ± 0.58</td>
</tr>
<tr>
<td>Choline:betaine</td>
<td>0.72 ± 0.16</td>
<td>0.75 ± 0.17</td>
<td>0.77 ± 0.18</td>
</tr>
<tr>
<td>Betaine:dimethylglycine</td>
<td>9.28 ± 2.90</td>
<td>10.7 ± 3.25</td>
<td>10.0 ± 2.74</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>21.9 ± 4.05</td>
<td>24.4 ± 7.48</td>
<td>22.3 ± 4.38</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)²</td>
<td>5.25 ± 1.35</td>
<td>5.27 ± 1.22</td>
<td>4.80 ± 1.29</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>203 ± 30.1</td>
<td>215 ± 30.7</td>
<td>211 ± 26.6</td>
</tr>
<tr>
<td>16 wk of gestation (n)</td>
<td>27</td>
<td>55</td>
<td>182</td>
</tr>
<tr>
<td>Choline (μmol/L)²</td>
<td>7.12 ± 1.74</td>
<td>6.70 ± 1.45</td>
<td>7.37 ± 1.93</td>
</tr>
<tr>
<td>Betaine (μmol/L)³</td>
<td>13.8 ± 4.41</td>
<td>12.7 ± 3.46</td>
<td>14.0 ± 4.37</td>
</tr>
<tr>
<td>Dimethylglycine (μmol/L)³</td>
<td>1.14 ± 0.50</td>
<td>0.99 ± 0.40</td>
<td>1.16 ± 0.49</td>
</tr>
<tr>
<td>Choline:betaine</td>
<td>0.54 ± 0.12</td>
<td>0.55 ± 0.13</td>
<td>0.55 ± 0.13</td>
</tr>
<tr>
<td>Betaine:dimethylglycine</td>
<td>13.6 ± 5.91</td>
<td>14.7 ± 7.07</td>
<td>13.4 ± 5.30</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>18.4 ± 3.66³</td>
<td>22.2 ± 4.99</td>
<td>21.4 ± 4.86</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)²</td>
<td>4.45 ± 1.13</td>
<td>4.50 ± 1.03</td>
<td>4.17 ± 1.48</td>
</tr>
<tr>
<td>Cysteine (μmol/L)</td>
<td>195 ± 34.0</td>
<td>210 ± 26.8</td>
<td>204 ± 32.5</td>
</tr>
</tbody>
</table>

¹ Blood was collected from 264 and 220 pregnant women at 16 and 36 wk of gestation, respectively, and women grouped by plasma total vitamin B-12.

² Significant difference between women with sufficient and marginal or deficient plasma total vitamin B-12, P < 0.05 (Student’s t test).

³ Mean ± SD (all such values).

⁴ Significant difference between women with deficient and sufficient plasma total vitamin B-12, P < 0.05 (1-factor ANOVA with Tukey’s honestly significant difference post hoc test).

⁵ Significant difference between women with marginal and sufficient plasma total vitamin B-12, P < 0.05 (1-factor ANOVA with Tukey’s honestly significant difference post hoc test).
Reduced plasma homocysteine is known to occur in pregnancy (34–39), is unrelated to folic acid supplementation, and is not explained by pregnancy-associated hemodilution or reduced plasma albumin (38). Similarly, hemodilution seems unlikely to explain the substantial 34–47% reduction in plasma total vitamin B-12 during pregnancy, and any hemodilution would have also blunted the apparent magnitude of increases in plasma free choline and dimethylglycine (Table 1). There are several adaptive changes in methionine metabolism in pregnancy (36), with a major role played by the complex endocrine changes that accompany pregnancy in the pregnancy-associated decrease in plasma homocysteine (38) and, perhaps, also in plasma methionine, cysteine, betaine, and dimethylglycine (Table 1).

Because of rising concerns over poor vitamin B-12 status in Canada (14–16) and data that have indicated that reduced vitamin B-12–folate–dependent homocysteine remethylation leads to increased remethylation by using betaine (3–5), we questioned if poor vitamin B-12 status occurs in pregnant women and limits the rise plasma free choline from midgestation to late gestation. Notably, our results showed that plasma free choline was higher in 36-wk-gestation women with a plasma total vitamin B-12 concentration $>220$ compared with $\leq 220$ pmol/L ($P < 0.05$), and the increase in plasma choline from midgestation to late gestation was also significantly correlated with plasma total vitamin B-12 at 36 wk of gestation ($P < 0.001$). A positive association between choline and vitamin B-12 could be explained by the reciprocal actions of the vitamin B-12–folate and choline-betaine-dimethylglycine pathways in homocysteine.

**FIGURE 3.** Scatter plots showing the relation between changes in plasma free choline from 16 to 36 wk of gestation and plasma tB12 at 36 wk of gestation ($n = 217$). Pearson’s correlation analysis was used to determine significant association between absolute and percentage increases in plasma free choline and the ln-transformed values for plasma tB12. tB12, total vitamin B-12.

**TABLE 4**

<table>
<thead>
<tr>
<th>Postnatal growth rates of infants of mothers grouped by plasma total vitamin B-12 at 36 wk of gestation$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal plasma total vitamin B-12</td>
</tr>
<tr>
<td>Boys</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
</tr>
<tr>
<td>2 mo (g · kg$^{-1}$ · d$^{-1}$)</td>
</tr>
<tr>
<td>6 mo (g · kg$^{-1}$ · d$^{-1}$)</td>
</tr>
<tr>
<td>9 mo (g · kg$^{-1}$ · d$^{-1}$)</td>
</tr>
<tr>
<td>Girls</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
</tr>
<tr>
<td>2 mo (g · kg$^{-1}$ · d$^{-1}$)</td>
</tr>
<tr>
<td>6 mo (g · kg$^{-1}$ · d$^{-1}$)</td>
</tr>
<tr>
<td>9 mo (g · kg$^{-1}$ · d$^{-1}$)</td>
</tr>
</tbody>
</table>

$^1$All values are means ± SDs. Birth weight and growth per day were calculated as [(weight – birth weight) (g)/birth weight (kg)/age (d)].

$^2$Significance between groups was determined by using multiple regression adjusted for maternal plasma total vitamin B-12 and breastfeeding (defined as fed <250 mL/wk human-milk substitute).
methylation, whereby the reduced activity of either pathway results in an increased activity of the other (Figure 1). However, plasma free choline was positively associated with methionine and cysteine, and no evidence of a decrease in plasma free choline:betaine or betaine:dimethylglycine with lower methionine:homocysteine ratios was shown. This may suggest that the positive association between plasma free choline, methionine, and cysteine and between total vitamin B-12 and free choline reflects increased choline synthesis. Possibly, higher vitamin B-12 status supports higher methionine-homocysteine recycling, which would also explain the positive association between plasma total vitamin B-12 and the methionine:homocysteine ratio in women in our study (Figure 2). However, phosphatidylcholine synthesized by the methylation of phosphatidylethanolamine has higher 20:4n-6 and 22:6n-3 than does phosphatidylcholine formed from diglyceride and CDP choline (25, 40). RBC phosphatidylcholine fatty acids showed no evidence of increased synthesis from phosphatidylethanolamine in women with deficient or marginal compared with sufficient plasma total vitamin B-12, although the deacylation-reacylation of phosphatidylcholine fatty acids postsynthesis could limit the usefulness of RBC phosphatidylcholine as a peripheral marker of altered methylation.

The Canadian Health Measures Survey (2007–2009) reported vitamin B-12 deficiency and marginal status (plasma total vitamin B-12 concentrations <148 and 148–220 pmol/L, respectively) in 5.0% and 20.3%, respectively, of adults 20–45 y of age (15). In contrast, folate deficiency is almost nonexistent in Canada and affected <1% of individuals surveyed (15, 41). Our dietary analyses showed vitamin B-12 intakes were significantly lower in pregnant women who met the criteria of total vitamin B-12 deficiency compared with sufficient (P < 0.01), with ~54% and 40% of dietary vitamin B-12 obtained from dairy products and meat plus fish, respectively. Although vitamin-supplement use was reported by all the pregnant women, supplemental forms of vitamin B-12 may be less bioavailable than natural sources, with availability also reduced when consumed with foods (42–44). The absence of dietary and supplement data for nonpregnant women was a limitation of our study. However, there was no reason to suspect that the lower plasma total vitamin B-12 in pregnancy is explained by higher vitamin B-12 intakes in nonpregnant than pregnant women. Consistent with national data, we showed no evidence of folate deficiency in any subject. However, 3% and 9% of the reference group of nonpregnant women met the criteria of deficient or marginal plasma total vitamin B-12, and these percentages increased to 10% and 21%, respectively, at 16 wk of gestation, with additional increases to 23% and 35%, respectively, at 36 wk gestation. The appropriateness of the extrapolation of reference values to assess vitamin B-12 status generated in nonpregnant adults for use in pregnancy has been questioned by other authors (31–34), and the use of our data to address this suitability is beyond the scope of the current study. However, the positive relation between plasma total vitamin B-12 and the methionine:homocysteine ratio (Figure 1) suggested the need to consider homocysteine recycling to regenerate methionine to support the increased methionine transmethylation that occur in pregnancy (36).

Post hoc analyses also gave evidence that maternal methyl nutrients may contribute to growth programming in apparently healthy term male infants. Our data showed significantly lower rates of weight gain during the first 6 mo after birth in infant boys but not girls born to women meeting the criteria of vitamin B-12 deficiency at 36 wk of gestation. Although no mechanisms are proposed from our work, other studies have reported sex-specific effects of gestational dietary variables on child growth as well as sex-specific effects of maternal methyl nutrients on offspring DNA methylation (45–47). Knowledge of the critical relation between perturbations in methyl metabolism in pregnancy and its relevance to human development is increasing (17, 45, 47, 48). Additional studies are needed to consider if the wide variability in choline status and its intersection with other methyl nutrients is of physiologic relevance to infant growth and development.

The authors’ responsibilities were as follows—SMI: study design, conception, data interpretation, and manuscript preparation; BTFW: compilation and analysis of data, data interpretation, and manuscript preparation; RAD and DJK: sample analysis; and KAM: data collection of infant growth and feeding. None of the authors had a conflict of interest.

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