Serum and red-blood-cell folate demonstrate differential associations with BMI in pregnant women

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Abstract

Objective: To examine the association between BMI and folate concentrations in serum and red blood cells (RBC) in pregnant women.

Design: A cross-sectional comparison of folate concentrations in serum and RBC sampled simultaneously from the same individual.

Setting: The Ottawa Hospital and Kingston General Hospital, Ontario, Canada.

Subjects: Pregnant women recruited between 12 and 20 weeks of gestation.

Results: A total of 869 pregnant women recruited from April 2008 to April 2009 were included in the final analysis. Serum folate was inversely associated and RBC folate positively associated with BMI, after adjusting for folic acid supplementation, age, gestational age at blood sample collection, race, maternal education, annual income, smoking and MTHFR 677C→T genotype. In stratified analyses, this differential association was significant in women with the MTHFR CC variant. In women with the CT and TT variants, the differential associations were in the same direction but not significant. Folic acid supplementation during pregnancy did not alter the differential association of BMI with serum and RBC folate concentration. This indicates that the current RBC folate cut-off approach for assessing risk of neural tube defects in obese women may be limited.

Conclusions: BMI is inversely associated with serum folate and positively associated with RBC folate in pregnant women, especially for those with the MTHFR CC variant.

Folate status in women of childbearing age is important due to its role in the prevention of neural tube defects (NTD). Previous studies have observed a differential association between obesity and serum and red-blood-cell (RBC) folate concentrations in men, non-pregnant women of childbearing age and postmenopausal women. Specifically, serum folate was found to be negatively associated, while RBC folate was positively associated, with BMI among non-pregnant adults. The mechanisms for this differential association of obesity with serum and RBC folate concentrations are not clearly understood. Some investigators have suggested that the altered pharmacokinetics and distribution of folate in obese persons may explain, at least in part, these differences. It was observed that the peak serum folate concentrations of obese and normal-weight women were statistically different after a single oral dose of folic acid (0.4 mg), but there was no difference in overall area under the curve, indicating that obesity does not impair the absorption of folic acid but may lead to redistribution of folate from circulation into tissue. Folate requirements are higher in pregnancy in order to maintain placental and fetal growth, and accelerated folate catabolism and haemodilution during pregnancy can also result in decreased circulating folate concentrations. Given the potential physiological and pharmacokinetic differences in pregnant persons,
the association of obesity with serum and RBC folate concentrations may be altered. Owing to the observed differential associations of folate with BMI in previous studies, the standard RBC folate cut-off approach for assessing NTD risk in obese women may be limited. Lower serum folate in obese women may reflect a redistribution of folate in response to the higher mass of maternal tissue, potentially limiting the amount of folate available to the developing embryo which accesses folate through serum folate rather than RBC folate, thus increasing the risk of NTD. The identification of subgroups of women who may be susceptible to low folate status or respond differently to folic acid supplementation will ensure that public health interventions can be tailored to meet the needs of the entire population. We therefore examined the association of BMI with serum and RBC folate concentrations in pregnancy using data collected in the Ottawa and Kingston (OaK) Birth Cohort study.

Method and materials

Data source
We used a subset of data from the OaK Birth Cohort, a prospective cohort study that recruited pregnant women at The Ottawa Hospital and Kingston General Hospital, Ontario, Canada, from September 2002 to April 2009. The subset included participants recruited from April 2008 to April 2009, from whom both serum and RBC folate were measured (Fig. 1). Participants were recruited between 12 and 20 gestational weeks and later delivered in the Ottawa–Carleton and Kingston regions. Participants were referred to the research team by clinic staff and asked to participate at their first antenatal visit. They were recruited into the study after information about the purpose of the study was provided and their written consent was obtained.

At the time of recruitment, participants’ self-reported demographic and lifestyle information including age, race, education, household income, folic acid supplementation and smoking. Nurses measured current height and weight at recruitment. Participants provided the brand name(s) of vitamin supplement(s) that were being consumed at the time of recruitment. Total daily supplemental folic acid intake was calculated based on the frequency of use and folic acid dose from all vitamin supplements. For women who were using more than one vitamin supplement, the total daily supplemental folic acid dose was calculated as the combined intake from all supplement sources.

Fasting blood samples were collected at the time of routine blood work, in order to avoid additional venepuncture. The blood samples were drawn from the antecubital vein or from the hand of the participating women at gestational age between 12 and 20 weeks, and measured in batches every month (i.e. the samples were stored for up to 1 month). Blood samples for serum folate testing were collected in serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and allowed to clot. Samples were centrifuged for 10 min at 3000g to separate serum. Serum was then removed and stored at −20°C until analysis. Folate concentrations were measured using the Beckman Coulter Access 2 and Unicel Dxl 800 immunoassay analysers using the manufacturer’s reagents (Beckman Coulter, Brea, CA, USA).

Blood samples for measuring RBC folate concentration were collected in 3 ml purple-top EDTA tubes. Haematocrit was determined in fresh samples using an OSM3 haemoximeter (Radiometer, Copenhagen, Denmark). Samples were frozen at −20°C until analysis, at which point they were thawed and cells were lysed using Access RBC Folate Lysing Agent (Beckman Coulter). RBC folate concentrations were quantified using the Beckman Coulter UniCel Dxl 800 Access Immunoassay System measured by competitive protein-binding assay. RBC folate concentrations were adjusted for haematocrit as described by the manufacturer.

Blood samples for genotyping the 677C→T polymorphism in the methylenetetrahyrofolate reductase gene (MTHFR) were collected in K₂EDTA Vacutainer tubes. Samples were centrifuged at 1100g and DNA was extracted using the BioRobot M48 and MagAttract DNA Blood Midi Kit (Qiagen, Hilden, Germany). PCR was used to amplify DNA regions of interest and genotyping was performed using the ABI 3130xd Genetic Analyzer and the ABI Prism SnaPshot Multiplex Kit (Applied Biosystems Inc., Foster City, CA, USA), which allows genotyping of several SNP in the same reaction. The laboratory procedures for genotyping have been described previously. For quality control, all assays were tested in hospital laboratories that are accredited by Ontario Laboratory Accreditation (OLA) and the Institute for Quality Management in Healthcare (IJQMH), and are ISO certified for quality, competence and beyond.

Statistical analysis
Biomarker concentrations were non-normally distributed; therefore the geometric mean was used in our analysis. In regression analyses, logarithmic transformations were applied to variables with skewed distributions.

BMI was calculated and categorized into normal and underweight (<25.0 kg/m²), overweight (25.0–29.9 kg/m²) and obese (≥30.0 kg/m²). Underweight cases (n 41) were combined with normal-weight cases due to limited sample size. Use of folic acid supplements was categorized as: (i) no supplement; (ii) supplement with folic acid intake ≤1 mg/d; and (iii) supplement with folic acid intake >1 mg/d.

Characteristics of the participants were described and compared across BMI categories using χ² tests. ANCOVA was used to examine folate concentrations associated with participant characteristics, including BMI, daily folic acid supplement intake, MTHFR 677C→T genotype (GG, CT and TT), maternal age, gestational age at blood sample collection, race, maternal education, household annual income and smoking.
Multiple linear regression analysis was used to examine associations of folate concentrations with BMI, in all samples and in subgroups stratified by MTHFR 677C→T genotype or folic acid supplementation. Interaction between BMI and genotype was tested in the overall model. Supplementary analysis using the pre-gravid weight (self-reported) was also performed. The level of significance was set at 0.05. Statistical analyses were performed using the statistical software package SAS version 9.2.

Results

A total of 889 participants who had complete records for both serum and RBC folate concentrations were identified in the cohort. Seven participants reported the use of vitamin supplements but did not include information on dose, and thirteen women missed information on MTHFR 677C→T genotype, leaving 869 participants for the final analysis (Fig. 1).

The overall geometric mean concentration of serum folate was 45.7 (SD 18.7) nmol/l and the geometric mean concentration of RBC folate was 1549 (SD 378) nmol/l. Serum folate <7.0 nmol/l was non-existent, and only one participant's serum folate was below 10 nmol/l. Demographic characteristics are shown in Table 1. Thirty-five participants (4%) were not taking any vitamin supplements. The proportion of overweight and obesity were 26% and 23%, respectively. Twelve per cent of participants were homozygous for the MTHFR 677C→T variant (TT). Gestational age at recruitment, education, annual income, folic acid supplementation and MTHFR 677C→T genotype were statistically different among the BMI categories. Women with BMI ≥ 30.0 kg/m² had a higher gestational age at recruitment, lower education, lower income, lower supplement use and were more likely to have the MTHFR CC variant.

Table 2 shows the determinants of serum and RBC folate concentrations. BMI showed an inverse association with serum folate but a positive association with RBC folate whether adjusted for or not (Table 2). Serum folate was lower with each additional variant allele such that CC > CT > TT; however, the trend was not statistically significant. Conversely, unadjusted and adjusted RBC folate was higher in participants homozygous for the T allele such that TT > CC and TT > CT. Folic acid supplementation was associated with higher serum and RBC folate concentrations. Gestational age at recruitment was a determinant of serum folate, and maternal age, gestational age at recruitment, education and smoking were determinants of RBC folate.

Table 3 shows the association between BMI and serum or RBC folate, for all participants and in subgroups stratified by MTHFR 677C→T genotype or folic acid supplementation, adjusted for demographic variables. Overall, serum folate was inversely associated with BMI and RBC folate was positively associated with BMI. Interaction between BMI and genotype was not significant (P>0.05) in the overall model (data not shown). When stratified by MTHFR genotype, serum folate was inversely associated with BMI in women with CC and CT variants, while RBC folate was positively associated with BMI in women with the CC variant. In women with the TT variant, both associations were insignificant, but the direction remained consistent with that observed for the CC and CT variants.

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**Fig. 1** Flowchart of participants, illustrating the procedure of study group identification (OaK, Ottawa and Kingston; GA, gestational age; RBC, red blood cells; MTHFR, methylenetetrahydrofolate reductase gene)

8085 participants recruited from September 2002 to April 2009

172 participants excluded from OaK Birth Cohort: GA beyond 12–20 weeks

6747 participants excluded from study subset: recruited before April 2008

Study subset: 1166 participants recruited from April 2008 to April 2009 for measurements of serum and RBC folate concentrations simultaneously

277 participants excluded: missing serum or RBC folate measurement

889 participants with available data on both serum and RBC folate measurement

20 participants excluded: 7 missed vitamin supplement dosage and 13 missed MTHFR 677C→T genotype

869 participants for final analysis
Table 1 Demographic characteristics of the study participants by BMI category: pregnant women (n 869) recruited between 12 and 20 weeks of gestation, Ottawa and Kingston (OaK) Birth Cohort study, April 2008–April 2009

<table>
<thead>
<tr>
<th>BMI (kg/m²)</th>
<th>Overall</th>
<th>&lt;25.0</th>
<th>25.0–29.9</th>
<th>≥30.0</th>
<th>P§</th>
</tr>
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<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>869</td>
<td>100.0</td>
<td>446</td>
<td>51.3</td>
<td>226</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>≤29</td>
<td>420</td>
<td>48.3</td>
<td>223</td>
<td>50.0</td>
<td>107</td>
</tr>
<tr>
<td>30–34</td>
<td>293</td>
<td>33.7</td>
<td>145</td>
<td>32.5</td>
<td>70</td>
</tr>
<tr>
<td>≥35</td>
<td>155</td>
<td>18.0</td>
<td>78</td>
<td>17.5</td>
<td>49</td>
</tr>
<tr>
<td>GA at recruitment (week)</td>
<td></td>
<td></td>
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<tr>
<td>12</td>
<td>584</td>
<td>67.2</td>
<td>308</td>
<td>69.1</td>
<td>158</td>
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<tr>
<td>13–15</td>
<td>239</td>
<td>27.5</td>
<td>113</td>
<td>25.3</td>
<td>55</td>
</tr>
<tr>
<td>16–20</td>
<td>46</td>
<td>5.3</td>
<td>25</td>
<td>5.6</td>
<td>13</td>
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<td>High school and below</td>
<td>170</td>
<td>19.6</td>
<td>91</td>
<td>20.4</td>
<td>34</td>
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<tr>
<td>College/university not completed</td>
<td>77</td>
<td>8.8</td>
<td>31</td>
<td>6.9</td>
<td>19</td>
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<tr>
<td>College/university completed</td>
<td>622</td>
<td>71.6</td>
<td>324</td>
<td>72.7</td>
<td>173</td>
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<tr>
<td>Race</td>
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<td>Caucasian</td>
<td>796</td>
<td>91.6</td>
<td>400</td>
<td>89.7</td>
<td>211</td>
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<tr>
<td>Other races</td>
<td>73</td>
<td>8.4</td>
<td>46</td>
<td>10.3</td>
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<td>Household annual income (SCAN)</td>
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<td>Declined</td>
<td>46</td>
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<td>31</td>
<td>6.9</td>
<td>6</td>
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<td>&lt;49 999</td>
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<td>128</td>
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<td>50 000–79 999</td>
<td>274</td>
<td>31.5</td>
<td>123</td>
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<td>≥80 000</td>
<td>305</td>
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<td>164</td>
<td>36.8</td>
<td>89</td>
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<tr>
<td>Smoking</td>
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<tr>
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<td>734</td>
<td>84.5</td>
<td>367</td>
<td>82.3</td>
<td>200</td>
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<tr>
<td>Yes</td>
<td>135</td>
<td>15.5</td>
<td>79</td>
<td>17.7</td>
<td>26</td>
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<tr>
<td>Folic acid supplement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-users</td>
<td>35</td>
<td>4.0</td>
<td>10</td>
<td>2.2</td>
<td>9</td>
</tr>
<tr>
<td>≤1 mg/d</td>
<td>710</td>
<td>81.7</td>
<td>377</td>
<td>84.6</td>
<td>183</td>
</tr>
<tr>
<td>&gt;1 mg/d</td>
<td>124</td>
<td>14.3</td>
<td>59</td>
<td>13.2</td>
<td>34</td>
</tr>
<tr>
<td>MTHFR 677C&gt;T genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>371</td>
<td>42.7</td>
<td>181</td>
<td>40.6</td>
<td>90</td>
</tr>
<tr>
<td>CT</td>
<td>394</td>
<td>45.3</td>
<td>212</td>
<td>47.5</td>
<td>112</td>
</tr>
<tr>
<td>TT</td>
<td>104</td>
<td>12.0</td>
<td>53</td>
<td>11.9</td>
<td>24</td>
</tr>
</tbody>
</table>

GA, gestational age; MTHFR, methylenetetrahydrofolate reductase gene.
†Data are presented as n and %, BMI = [weight (kg)]/[height (m)]². BMI was measured in pregnancy (between 12 and 20 weeks of gestation); this may lead to misclassification of BMI category.
§P value of χ² test.
‖Folic acid from supplements, not including folic acid from fortified foods or naturally occurring food folate.

Supplementary analysis using self-reported pre-gravid weight yielded similar results (data not shown).

Discussion

Our study confirmed a differential association of BMI with serum and RBC folate concentrations in pregnant women, such that BMI and serum folate concentration had an inverse relationship, and BMI and RBC folate concentration had a positive relationship. The differential associations were not significant in women with CT or TT genotype, although the direction of these associations was consistent in women with MTHFR CC genotype. Our data indicate that the differential association between serum and RBC folate and BMI observed in non-pregnant obese women likely persists into pregnancy. Serum folate was also found to be inversely associated with BMI at mid and late pregnancy in Korean women, although no RBC folate was measured simultaneously in that study(14). In both Canadian and American populations, non-pregnant obese women, including those of childbearing age, have been shown to have lower plasma folate and higher RBC folate in comparison to normal-weight women(2,5,7). These associations remained even after adjustment for dietary and supplemental folate intake, an important consideration given the observed pattern of lower folate intake among obese women(2,5).

The associations were significant only for women with CC genotype and not significant for those with TT owing to limited sample size in the TT group, as well as increased measurement error attributed to the use of a competitive protein-binding assay to measure RBC folate, which is an important limitation of the present study. We observed a CC > CT > TT pattern in serum folate concentration, but a reversed TT > CC and TT > CT pattern for RBC folate concentration. In a meta-analysis, Tsang et al. reported a CC > CT > TT pattern in serum and RBC folate concentrations measured by microbiological assay, which was reversed for RBC folate concentrations measured by protein-binding assay(15). The differences between the assays are due to differences in affinity of the assays for...
various folate forms. Plasma folates are exclusively monoglutamates and the majority are in the form of 5-methyltetrahydrofolate, whereas the majority of RBC folates are variable-length, long-chain polyglutamate 5-methyltetrahydrofolate. RBC folates must be hydrolysed into the monoglutamate form. The differences in affinities among these folate forms, as well as inconsistent proportion of folate forms among those with MTHFR 677C→T homozygous genotype (TT), impair the interpretation of RBC folate concentration measured by protein-binding assay(16).

There are several other limitations to our study. First, the study design is cross-sectional, and serum and RBC folate were measured in early pregnancy; therefore, it is not possible to determine whether these associations hold through late pregnancy when haemodilution could have a significant effect. Second, the type and dose of supplements were self-reported and may be subject to measurement error due to inaccurate patient report and irregular pattern of use. We did not conduct dietary surveys on participants. Previous studies reported that obese women tended to under-report their food intake(17,18), and folate from naturally occurring food sources exhibits variable and incomplete bioavailability, which can be affected by physiological conditions, pharmaceuticals and genetic polymorphisms(19–21). Third, BMI was measured in early pregnancy (95% were measured before 15 weeks) and this may lead to misclassification of obesity, although the weight gain in early second trimester is generally small(22). Finally, samples were measured in batches and the storage
time varied, but was no longer than 1 month. Serum folate stored in a frost-free freezer at −20°C for even a short period may be relatively unstable and sensitive to minor temperature fluctuations (25). This may contribute to the variation in folate concentrations.

There are several strengths of the present study. First, to our knowledge, it is the first study to examine the association of obesity with serum and RBC folate concentrations in pregnant women. Because of the difference in folate metabolism between the non-pregnant and pregnant populations, it is important to examine if the observed differential association between obesity and folate concentrations measured from serum vs. RBC would persist in a pregnant population (14–16). Second, our study was based on data collected in a prospective cohort, which mitigates recall bias. Third, serum folate and RBC folate concentrations in our study were derived from the same fasted participants, thereby mitigating bias.

The placenta takes up folate from the maternal plasma by the reduced folate carrier, the proton-coupled folate transporter and the folate receptor (27,28). Placental uptake of circulating folate ensures an adequate folate supply to the developing fetus. Obese women are at increased risk for NTD-affected pregnancy (29–32). A proportion of the NTD risk associated with maternal obesity may be due to lower circulating folate in obese women, which can be corrected to some degree with higher folate intake (29). On the other hand, it must be noted that a proportion of the NTD risk associated with obesity appears to be independent of folate status and may be due to other metabolic disturbances (33). Obese women of childbearing age may therefore require supplemental folic acid to increase or maintain a plasma folate status that is associated with maximal protection from NTD. However, folate status associated with NTD protection is currently expressed in terms of RBC folate (34). Because of the observed differential association of RBC folate with BMI, the standard RBC folate cut-off approach for assessing NTD risk in obese women may be limited. The relationship between RBC folate and serum folate, and how they relate to NTD risk reduction, may need to be determined for women of all BMI categories to determine appropriate ‘cut-offs’ for NTD protection.

A second consideration is whether obese women respond to folic acid supplementation similar to normal-weight women. Folic acid supplementation did not necessarily mitigate the observed differential association of BMI with serum and RBC folate in these pregnant women. A pharmacokinetic study found that obese women demonstrated a lower peak serum folate concentration after a 400 µg bolus folic acid dose and a lower area under the curve in the absorptive phase (0–3 h post-bolus), but no difference in the overall area under the curve (27). The data indicate that obesity may delay the absorption of folic acid but that overall absorption is not impaired. It should be noted that the women in our study had a folate status

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Table 3  Association of BMI with serum and RBC folate, stratified by MTHFR 677C→T genotype and folic acid supplementation; pregnant women (n = 869) recruited between 12 and 20 weeks of gestation, Ottawa and Kingston (OaK) Birth Cohort study, April 2008–April 2009

<table>
<thead>
<tr>
<th></th>
<th>Serum folate‡</th>
<th>RBC folate‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>95% CI</td>
</tr>
<tr>
<td>All participants§</td>
<td>-0.32</td>
<td>-0.50, -0.16</td>
</tr>
<tr>
<td>MTHFR 677C→T genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>-0.42</td>
<td>-0.66, -0.17</td>
</tr>
<tr>
<td>CT</td>
<td>-0.25</td>
<td>-0.49, -0.02</td>
</tr>
<tr>
<td>TT</td>
<td>-0.16</td>
<td>-0.44, 0.12</td>
</tr>
<tr>
<td>Folic acid supplementation‖</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-users</td>
<td>-0.62</td>
<td>-1.09, -0.14</td>
</tr>
<tr>
<td>≤1 mg/d</td>
<td>-0.32</td>
<td>-0.48, -0.16</td>
</tr>
<tr>
<td>&gt;1 mg/d</td>
<td>-0.21</td>
<td>-0.78, 0.37</td>
</tr>
</tbody>
</table>

RBC, red blood cell; MTHFR, methylenetetrahydrofolate reductase gene; β, the partial regression coefficient of log-transformed BMI.
‡BMI, serum folate and RBC folate concentrations were log-transformed.
§Regression coefficient of BMI in all participants, adjusted for race, maternal education, household annual income and smoking.
‖Regression coefficient of BMI in subgroups stratified by MTHFR 677C→T genotype or folic acid supplementation, adjusted for age, gestational age at recruitment, race, maternal education, household annual income and smoking.
associated with maximal protection from NTD risk \((\geq 906 \text{nmol/l})\)\(^{35}\). In our study, the overall mean RBC folate was 1549 (so 378) nmol/l and 1275 (95% CI 1155, 1409) nmol/l in supplement non-users. This is comparable to findings from the Canadian Health Measures Survey (2007–2009) where mean RBC folate (also measured by immunoassay) was 1142 (95% CI 1153, 1239) nmol/l in women of childbearing age who did not consume a folic acid supplement\(^{30}\). These findings are reflective of the mandatory folic acid fortification of white wheat flour in Canada. The relationship of obesity with folate status may be more of a concern in countries where fortification is not widespread and supplement use is lower.

The differential association of obesity with serum folate concentration \(v\). RBC folate concentration observed in the present study deserves attention in research and perinatal health practice alike. The identification of subgroups of women who may be susceptible to low folate status or respond differently to folic acid supplementation will ensure that public health interventions can be tailored to meet the needs of the entire population. Obesity is a known risk factor for a number of adverse maternal and infant outcomes including pre-eclampsia\(^{37,38}\), gestational diabetes\(^{39,40}\) and NTD\(^{41}\). Folic acid deficiency is a known risk factor for NTD\(^{42,43}\) and may be associated with other pregnancy complications such as pre-eclampsia\(^{44,45}\) and other congenital anomalies\(^{46,47}\). As a result, in the study of the association between folate status and adverse maternal and infant outcomes, it is critical to use the appropriate biological specimen for the measurement of folate. Since BMI is positively associated with RBC folate but inversely associated with serum folate, the observed associations between folate status and pregnancy complications may be overestimated if serum folate is used while underestimated if RBC folate is used. Folate status varies substantially during pregnancy\(^{48}\), which can further complicate the accurate assessment of folate status.

In summary, we confirmed the differential associations of folate with BMI in pregnant women, indicating that the RBC folate cut-off approach for assessing NTD risk may be limited. Because absorption and metabolism of folate are modified by adiposity, RBC folate may not reflect freely available folate at cellular level in pregnant women.

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