Maternal serum folate species in early pregnancy and risk of preterm birth\(^1\textsuperscript{-3}\)

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ABSTRACT

Background: Poor maternal folate status has been associated with an increased risk of preterm birth. However, major gaps remain in our understanding of how individual folate species relate to preterm birth.

Objective: Our objective was to assess the association between maternal folate status as measured by 5-methyltetrahydrofolate (5MeTHF), 5-formyltetrahydrofolate (5FoTHF), and folic acid concentrations, which are the 3 primary folate species in serum, and the risk of preterm birth and spontaneous preterm birth (sPTB).

Design: A cohort of 313 pregnant women who received care at resident antepartum clinics at Magee-Womens Hospital (Pittsburgh, PA) (2003–2007) was enrolled at \(<16\) wk gestation. We analyzed nonfasting blood samples that were drawn from subjects at enrollment for the 3 folate species by using HPLC–tandem mass spectrometry.

Results: Serum 5MeTHF and 5FoTHF concentrations comprised 65\% and 33\% of total folate concentrations, respectively. In confounder-adjusted, multivariable, log-binomial regression models, 1-SD increases in serum total folate and serum 5MeTHF concentrations were associated with significant reductions in the risk of sPTB \((P < 0.05)\). There was a significant interaction between serum 5MeTHF and 5FoTHF concentrations and risk of preterm birth \((P = 0.01)\). When serum 5MeTHF concentrations were low, there was a positive linear relation between 5FoTHF and risk of preterm birth. When 5MeTHF concentrations were high, there was a strong negative relation between 5FoTHF and preterm birth.

Conclusions: Our results imply that the relative concentrations of folate species may be more critical than total folate in preventing preterm birth. An improved understanding of folate metabolism during pregnancy may lead to targeted intervention strategies that decrease the rate of preterm birth. Am J Clin Nutr 2010;92:864–71.

INTRODUCTION

The prevention of preterm birth is a major public health priority \((1)\). Infants born preterm (ie, \(<37\) wk of gestation) are more likely than full-term infants to die as neonates or infants and to suffer from serious short- and long-term morbidities, including cerebral palsy, mental retardation, language, learning, and behavior problems, and poor health and growth \((2)\). In the United States, 1 in 8 infants is born preterm, and in 2005, preterm births cost the United States \(\$26\) billion \((2)\). Unfortunately, interventions aimed at preventing preterm birth have been disappointing \((2)\).

Maternal folate status is a modifiable factor that may be linked with preterm birth. Studies of dietary, supplemental, or biomarker folate in relation to preterm birth have produced conflicting results \((3–14)\) that were likely due to varying ranges and timing of folate assessment, assessment methods of gestational age, and population characteristics. It is particularly critical to reexamine the association between folate intake and preterm birth after the nationwide 1998 implementation of the program to fortify enriched cereal-grain products with folic acid \((15)\), which substantially improved the folate status of US childbearing-aged women \((16)\). From 1988–1994 to 2003–2004, serum folate concentrations of women aged 15–45 y doubled, and the prevalence of deficiency dropped from 21\% to \(<1\% \((16)\).

New investigations are needed to delineate the role of individual folate metabolites related to preterm birth. The folate metabolic pathway is critical for DNA synthesis and 1-carbon metabolism that leads to methylation \((\text{Figure 1})\). The 3 primary folate species in serum are 5-methyltetrahydrofolate \((5\text{MeTHF})\), 5-formyltetrahydrofolate \((5\text{FoTHF})\), and folic acid. The different folate species mediate the varied biological effects of folate. Studies that investigate specific folate species’ association with preterm birth can improve current nutrition intervention strategies and give insights into biological pathways that influence the risk of preterm birth. Our objective was to assess the independent association between first-trimester maternal folate status as measured by serum 5MeTHF, 5FoTHF, and folic acid concentrations and the risk of preterm birth.

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SUBJECTS AND METHODS

Data came from the Study of Nutrition and Pregnancy (SNAP), an ongoing prospective cohort study of pregnant women seeking care at resident antepartum clinics at Magee-Womens Hospital (Pittsburgh, PA). The antepartum clinics serve a predominantly publically insured, low-income population that is 55% black and 44% white. Eligible women had singleton pregnancies, were non-Hispanic whites or non-Hispanic blacks on the basis of self-reports, and had no known preexisting conditions, vaginal bleeding, fetal anomalies, or current or planned cervical cerclage. Enrollment took place at 16 wk of gestation (mean gestational age: 9.5 wk) after women provided informed, written consent. The study was approved by the University of Pittsburgh Institutional Review Board.

At enrollment, women completed an interviewer-administered questionnaire to collect data on sociodemographic characteristics, medical, reproductive, and sexual history, and maternal behaviors. Also at enrollment, women provided a nonfasting blood sample that was banked for later analysis. A total of 546 eligible women enrolled in the study from June 2003 to December 2007 (75% response rate). Of these women, we excluded 163 women because they had a spontaneous or therapeutic abortion (n = 67), transferred care (n = 50), delivered a stillbirth (n = 5), were lost to follow-up (n = 31), or lacked data on covariates in the final model (n = 11). An additional 69 women were excluded because they did not have an adequate volume of banked serum to perform the folate assays. There were no meaningful differences in maternal characteristics or in the prevalence of preterm birth between women included and excluded from the analyses (data not shown). A total of 313 women were included in the final analyses.

Gestational age was based on the best obstetrical estimate from a reliable, self-reported estimate of last menstrual periods (LMPs) or ultrasounds early in pregnancy. Preterm birth was defined as a delivery of a live infant that occurred at <37 completed weeks of gestation. Spontaneous preterm birth (sPTB) was defined as a preterm delivery that occurred after preterm labor with intact membranes or a preterm prelabor rupture of the fetal membranes.

Quantification of folate species

Nonfasting serum samples of subjects were allowed to coagulate at room temperature for 30 min. Subsequently, tubes were kept on ice and covered with foil to protect them from light. Within 2 h of the blood draw, tubes were centrifuged, and serum was aliquotted into amber vials. Vials were stored at −80°C until they were transported on dry ice in 2009 to the local laboratory of Raman Venkataramanan. No samples were thawed before assay for folate species.

Folate species were quantified with high-pressure liquid chromatography–tandem mass spectrometry (LC-MS/MS) on the basis of a published method (17). The standard curve was prepared by using a mixture of standard folates added to blank human plasma from which endogenous folates were removed by treating the mixture with activated charcoal. Each analyte included was prepared at 0, 1.8, 4.5, 9.0, 13.5, 22.5, and 54 ng/mL. A total of 25 μL internal standard (1 μL of a mixture of 13C5-5MeTHF, 13C6-5FoTHF and 13C6-FA) was added to 400 μL standard samples and/or 400 μL patient plasma samples, and samples were diluted with 0.9 mL solid-phase extraction sample buffer (10 g ammonium formate/L, 1 g ascorbic acid/L; pH 3.2). After a 20-min equilibration at 4°C, the diluted plasma sample was passed through Bond Elut PH 1-cc (100 mg) extraction cartridges (Varian, Palo Alto, CA) under vacuum. These columns were preconditioned with 2 mL methanol and 1 mL solid-phase extraction sample buffer (10 g ammonium formate/L and 1 g ascorbic acid/L; pH 3.2). After washing with 1 mL wash buffer (0.5 g ammonium formate/L and 0.05 g ascorbic acid/L; pH 3.4) and 150 μL elution buffer (400 mL methanol/L, 100 mL acetonitrile/L, 10 mL acetic acid/L, and 1 g ascorbic acid/L), folates were eluted with 150 μL elution buffer, and 20 μL of solution was injected into the high-pressure liquid chromatograph piloted by MassLynx 4.1 software (Waters Inc, Milford, MA).
Chromatography was conducted with a C8 4 × 2.0-mm guard column and a Luna 5-μm C8 (2) 150 × 3.0-mm analytic column (Phenomenex, Torrance, CA) that was maintained at 30°C by using isocratic elution with a mobile phase that consisted of 400 mL methanol/L, 100 mL acetonitrile/L, and 10 mL acetic acid/L at flow rate of 0.25 mL/min. Analyses were conducted with a Waters Quattro micro atmospheric pressure ionization mass spectrometer (Waters Inc) with positive electrospray ionization made using multiple reaction monitoring. The ratios of the peak areas of 5MeTHF, 5FoTHF, and folic acid (beled internal standard were linearly related to the concentrations action monitoring. The ratios of the peak areas of 5MeTHF, 5FoTHF, and folic acid/L at flow rate of 0.25 mL/min.

Using isocratic elusion with a mobile phase that consisted of (Phenomenex, Torrance, CA) that was maintained at 30°C (176°C) was calculated according to the methods of Flanders and Khoury (23). We compared the RRs (95% CIs) from the conventional logistic regression model of total folate and preterm birth risk with estimates obtained from the sensitivity-analysis iterations, which reflected systematic error and random error associated with missing data on regular multivitamin use (23). Stata Software, version 10 (StataCorp, College Station, TX) and SAS software, version 9.2 (SAS, Cary, NC) were used for analyses.

RESULTS

The majority of women in the cohort were 20–29 y old, multiparous, high-school educated, unmarried, unemployed, and had low-incomes (Table 1). Approximately one-half of the women were non-Hispanic blacks, and one-half of the women were smokers. Over 60% of women were overweight or obese. The median (interquartile range) gestational age at enrollment was 9.4 wk (7.5–12.1 wk). Mothers in the highest third of the folate distribution (tertile 3) were significantly more likely to be non-Hispanic white, nulliparous, nonsmokers, and married than were mothers in the other groups.

None of the women were folate deficient (serum total folate concentration <3 ng/mL) (26). The medians (interquartile ranges) for serum total folate, 5MeTHF, and 5FoTHF concentrations were 24.7 (19.0–34.3), 15.8 (11.6–21.9), and 7.8 (4.7–12.5) ng/mL, respectively (Figure 2A). Serum 5MeTHF and 5FoTHF concentrations made up 65% (interquartile range: 53–77%) and 33% (interquartile range: 21–45%) of total folate concentrations, respectively. There was a weak, but significant linear positive association between serum 5MeTHF and 5FoTHF concentrations (r = 0.15, P < 0.01; Figure 3). Only 8% of women (n = 21) had detectable serum folic acid concentrations, and among these women, serum folic acid contributed from 3.5 to 53% of their total folate concentrations. The distributions of folate species by total folate tertile are shown in Figure 4.
There were 40 cases of preterm birth (12.8%), including 27 cases of sPTB (9.0%), in the cohort. There were no significant differences in unadjusted median folate concentrations by preterm birth status (Figure 2, B and C). However, after adjustment for race-ethnicity, education, smoking, and obesity, women in the highest tertile of total folate concentrations had a 60% reduction in the risk of preterm birth [RR (95% CI): 0.4 (0.1, 0.9); Table 2]. The results were less precise and included the null value after accounting for unmeasured confounding by regular periconceptional multivitamin use [point estimate (95% sensitivity analysis interval): 0.4 (0.1, 1.0)]. The association between the highest 5MeTHF concentration tertile and preterm birth was of borderline statistical significance (P = 0.08). Importantly, we observed a significant interaction between serum 5MeTHF and 5FoTHF concentrations on the risk of preterm birth (P = 0.01; Figure 5). When serum 5MeTHF concentrations were low (8.7 ng/mL; 10th percentile), there was a positive linear relation between 5FoTHF and risk of preterm birth. Indeed, the RRs (95% CIs) for 5FoTHF concentrations of 7.8, 12.2, and 18 ng/mL were 1.4 (1.1, 1.9), 1.9 (1.1, 3.4), and 2.7 (1.1, 7.4), respectively, compared with the referent of 2.8 ng/mL, in women with low 5MeTHF concentrations. When serum 5MeTHF concentration was at the median of the distribution, no relation was observed with preterm birth regardless of the serum 5FoTHF concentration. In contrast, when 5MeTHF concentrations were high (27.9 ng/mL; 90th percentile), there was a strong negative relation between 5FoTHF and preterm birth. Concentrations of 5FoTHF of 12.2 and 18 ng/mL combined with concentrations of 5MeTHF of 27.9 ng/mL were protective against preterm birth [RR (95% CI): 0.33 (0.11, 0.97) and 0.15 (0.03, 0.78), respectively].

When we studied sPTB as the outcome, serum total folate concentrations in the highest tertile were associated with an 80% reduction in risk of sPTB [RR (95% CI): 0.2 (0.1, 0.8)] compared with the bottom tertile after confounder adjustment (Table 2). In fact, when total folate was entered into the model as a continuous variable, there was a linear relation between total folate concentrations and the risk of sPTB. A 1-SD increase in serum total folate concentrations was associated with a 40% reduction in sPTB. The relations between serum total folate concentrations and sPTB were driven by serum 5MeTHF concentrations, as
evidenced by the similar sPTB adjusted RR. We did not observe an interaction between 5MeTHF and 5FoTHF concentrations and the risk of sPTB.

**DISCUSSION**

Despite the absence of clinical folate deficiency in our cohort, we observed negative linear associations between total folate concentrations and the risk of sPTB as well as between 5MeTHF concentrations and the risk of sPTB. We also observed an interaction between 5MeTHF and 5FoTHF concentration and the risk of preterm birth, whereby low concentrations of 5MeTHF and high concentrations of 5FoTHF increased the risk, whereas high concentrations of 5MeTHF and 5FoTHF reduced the risk. These relations were shown in a cohort recruited after mandated folic acid fortification of grain products in the United States. To our knowledge, this is the first study of individual folate species in relation to preterm birth occurrence. Our results on total serum folate concentrations are consistent with those shown in other pregnancy cohorts. For instance, in 2468 North Carolina women, Siega-Riz et al (3) observed that serum total folate concentrations <16.3 ng/mL (which corresponds to our lower tertile of total folate), red cell total folate concentrations ≤627 ng/mL, and intake of ≤500 µg dietary folate/d at 24–28 wk was associated with 50–90% increases in preterm birth and sPTB. In a low-income group of 832 New Jersey mothers, Scholl et al (4) showed a negative linear relation between serum total folate concentrations at 28 wk and preterm birth and an elevated risk of preterm birth with midpregnancy folate intake of ≤400 µg folate/d. These results are supported by 2 randomized trials of folic acid plus iron compared with iron alone that were started in late pregnancy, which showed an increased mean gestational age with folic acid and iron treatment (5, 7).

In one of the few studies published that used a cohort post–folic acid fortification, 70% and 50% reductions in the risk of sPTB at 20 to <28 and 28 to <32 wk, respectively, were shown in women who reported using preconceptional multivitamins containing folic acid for ≥1 y (27). Results were similar in 2 other studies of preconceptional (28) and periconceptional (29) folic acid–containing multivitamin use. However, because these researchers combined individuals who used folic acid alone or folic acid in a multivitamin, it is impossible to determine whether the risk reduction was a result of folate or other micronutrients taken with folate.

In contrast, no relation between folate intake and preterm birth was shown in several observational investigations of folate biomarkers (8–10) and randomized trials of folic acid supplementation (11–14). However, it is notable that these studies relied solely on the mother’s report of the first day of her LMP to determine gestational age at delivery. LMP-derived gestational
age is error prone because mothers may not perfectly recall their LMP and may misidentify a LMP that was due to postconception bleeding, delayed ovulation, or intervening early miscarriage. A Hungarian randomized trial of folic acid–containing multivitamins compared with trace elements only taken before conception to 12 wk gestation reported no effect on preterm birth (30), but the supplementation may have ended too early to have seen effects on the length of gestation, and folic acid was not studied alone. Despite these negative studies, the preponderance of evidence suggests that improved folate status is associated with a reduced risk of preterm birth.

Although a growing body of literature implicates an effect of folate in preterm birth, little is known about the underlying mechanisms. Folate is central to a variety of complex biological processes including purine and thymidine synthesis, DNA methylation, and metabolism of homocysteine. The different folate species are involved preferentially in these distinct biological pathways. For example, 5MeTHF, by using vitamin B-12 as a cofactor, donates a methyl group to convert homocysteine to methionine. Methionine is necessary for protein biosynthesis and the generation of S-adenosyl methionine (SAM). SAM is critical in DNA methylation. In contrast, 5FoTHF is central to many biological pathways including purine and pyrimidine synthesis. Our data of the interaction between 5MeTHF and 5FoTHF suggest that certain biological pathways may be more critical for determining preterm birth risk.

Our finding of the interaction between 5MeTHF and 5FoTHF concentrations on preterm birth also suggests that the balance of these species, rather than the total folate concentration, has important biological consequences. This observation highlights the need to understand the determinants of the relative concentrations of folate species and, thus, the risk of preterm birth. Engel et al (31) showed that a gene-sequence variation in the SHMT1 gene, which is associated with less serine hydroxymethyltransferase 1 transcriptional activity, interacted with a low dietary intake of folate to increase odds of a sPTB. Our findings provide some biologic corroboration to these results. A second important area of investigation is the effect of different sources of folate on the proportion of folate species. It is possible that folate derived from supplements compared with from diets could contribute differentially to the relative proportion of folate species. Unfortunately, our collection of dietary and supplement use data in this ongoing cohort study began after 80% of women in this study were enrolled, so we do not have adequate data to understand the determinants of folate species in our population.

The folate status of our population was similar to that of the National Health and Nutrition Examination Survey 1999–2004 that was based of women of all ages (16) after application of the equations to convert the Bio-Rad radioassay results to LC-MS/MS (32). Also, compared with others (17), we observed smaller contributions of 5MeTHF and folic acid and larger contributions of 5FoTHF to total folate concentrations. Folic acid was observed in serum after recent consumption of >300 μg folic acid from supplements or fortified food (33). Data on oral intake of folic acid from our population is needed to understand these low concentrations. Although some investigators have suggested that 5FoTHF in serum actually may be 5MeTHF that has degraded as a result of improper handling of samples (34), our careful data analysis suggests that the relative concentrations of these species are critical to determining the risk of preterm birth.
sample management makes this explanation unlikely. There may be an alteration in folate metabolism during pregnancy to cause this increase in 5FoTHF concentrations, but we are not aware of studies that have addressed this issue (33).

Our study was limited by a lack of data on folate intake and measurements of other key metabolites in the folate pathway, including homocysteine, vitamin B-12, methionine, SAM, and S-adenosylhomocysteine. Serum folate concentrations do not represent long-term folate status and may be affected by recent folate intake, given that our samples were nonfasting. As with all observational studies, our study can only suggest associations, not causation. We attempted to account for factors that are associated with folate status and preterm birth, including regular periconceptional multivitamin use (an unmeasured confounder). Nevertheless, we cannot exclude the possibility that confounding by additional unmeasured factors, such as physical activity, family support, psychosocial stress, household conditions, and genetic factors, may have biased our results, and confounders we did collect may have been measured with error. Our sample size limited our ability to study subtypes of preterm birth (eg, early compared with late preterm birth), which may provide more evidence into mechanisms by which folate may protect

### TABLE 2

Association between tertiles of folate species at <16 wk and risk of preterm birth (PTB) and spontaneous preterm birth (sPTB)

<table>
<thead>
<tr>
<th></th>
<th>Term PTB</th>
<th>Adjusted RR (95% CI)</th>
<th>Term sPTB</th>
<th>Adjusted RR (95% CI)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
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<tr>
<td><strong>Total folate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (16.8 ng/mL)</td>
<td>89 (86)</td>
<td>15 (14)</td>
<td>1.0 (ref)</td>
<td>89 (88) 12 (12) 1.0 (ref)</td>
</tr>
<tr>
<td>T2 (24.7 ng/mL)</td>
<td>86 (82)</td>
<td>19 (18)</td>
<td>1.3 (0.7, 2.4)</td>
<td>86 (88) 12 (12) 1.0 (0.5, 2.2)</td>
</tr>
<tr>
<td>T3 (38.9 ng/mL)</td>
<td>98 (94)</td>
<td>6 (6)</td>
<td>0.4 (0.1, 0.9)</td>
<td>98 (97) 3 (3) 0.2 (0.1, 0.8)</td>
</tr>
<tr>
<td>1-SD (12-ng/mL) increase</td>
<td>0.7 (0.5, 1.0)</td>
<td></td>
<td></td>
<td>0.6 (0.4, 0.9)</td>
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<tr>
<td><strong>5MeTHF</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T1 (10.2 ng/mL)</td>
<td>89 (84)</td>
<td>17 (16)</td>
<td>1.0 (ref)</td>
<td>89 (86) 14 (14) 1.0 (ref)</td>
</tr>
<tr>
<td>T2 (15.8 ng/mL)</td>
<td>90 (86)</td>
<td>15 (14)</td>
<td>0.9 (0.5, 1.7)</td>
<td>90 (91) 9 (9) 0.7 (0.3, 1.5)</td>
</tr>
<tr>
<td>T3 (25.1 ng/mL)</td>
<td>94 (92)</td>
<td>8 (8)</td>
<td>0.5 (0.2, 1.1)</td>
<td>94 (96) 4 (4) 0.3 (0.1, 0.8)</td>
</tr>
<tr>
<td>1-SD (7.9 ng/mL) increase</td>
<td>0.8 (0.5, 1.1)</td>
<td></td>
<td></td>
<td>0.6 (0.4, 0.9)</td>
</tr>
<tr>
<td><strong>5FoTHF</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>T1 (3.7 ng/mL)</td>
<td>90 (87)</td>
<td>13 (13)</td>
<td>1.0 (ref)</td>
<td>90 (94) 6 (6) 1.0 (ref)</td>
</tr>
<tr>
<td>T2 (7.8 ng/mL)</td>
<td>91 (88)</td>
<td>13 (12)</td>
<td>1.1 (0.5, 2.2)</td>
<td>91 (88) 13 (12) 2.0 (0.8, 5.2)</td>
</tr>
<tr>
<td>T3 (15.5 ng/mL)</td>
<td>92 (87)</td>
<td>14 (13)</td>
<td>1.2 (0.6, 2.4)</td>
<td>92 (92) 8 (8) 1.3 (0.5, 3.6)</td>
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<tr>
<td>1-SD (12 ng/mL) increase</td>
<td>0.9 (0.7, 1.2)</td>
<td></td>
<td></td>
<td>0.9 (0.7, 1.14)</td>
</tr>
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</table>

1. T1, bottom tertile; T2, middle tertile; T3, upper tertile; RR, risk ratio; ref, reference; 5MeTHF, 5-methyltetrahydrofolate; 5FoTHF, 5-formyltetrahydrofolate.
2. Adjusted for race-ethnicity, education, smoking, and obesity.
3. Adjusted for education and smoking.
4. Values in parentheses are median values for the tertile.
5. \( P < 0.05 \) (test of trend).

### FIGURE 5

Interaction between maternal serum 5-methyltetrahydrofolate (5MeTHF) and 5-formyltetrahydrofolate (5FoTHF) on the risk of preterm birth \((n = 313; P = 0.01)\). Serum 5MeTHF concentrations were chosen at the 10th, 50th, and 90th percentiles of the distribution, and 5FoTHF concentrations were chosen at the 10th, 25th, 50th, 75th, and 90th percentiles of the distribution. Risk ratios (RRs) were generated on the basis of linear combinations of the log-binomial regression-model coefficients by using the concentrations of 5FoTHF and 5MeTHF provided. All RRs were adjusted for race-ethnicity, smoking before pregnancy, education, and obesity.
against early delivery. Nonetheless, our study’s prospective design, rigorously defined preterm-birth phenotype, and quantification of 3 folate metabolites with the gold-standard method of LC-MS/MS are major strengths.

In conclusion, the prevalence of preterm birth has risen steadily over the past 20 y (35), with no decline after the nationwide folic acid fortification of grain products. If others confirm our results, the relative concentrations of folate species may prove more critical than total folate concentrations in preventing preterm birth. Furthermore, these data may be important in the design of clinical trials aimed at determining the optimal composition of folate supplements and food fortification. An improved understanding of folate metabolism during pregnancy may lead to targeted intervention strategies that decrease the rate of preterm birth.

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The authors’ responsibilities were as follows—LMB and HNS: designed the research; RV and HNS: provided the data; LMB and J-YC: analyzed data; JLM: conducted the literature review; LMB, KPH, and HNS: wrote the manuscript; and J-YC, VR, RWE, and JLM: provided significant advice. All authors read and approved the final manuscript. None of the authors had a personal or financial conflict of interest.

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