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Supplementation with (6*S*)-5-methyltetrahydrofolic acid appears as effective as folic acid in maintaining maternal folate status while reducing unmetabolised folic acid in maternal plasma: a randomised trial of pregnant women in Canada

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Abstract

Folic acid supplementation is recommended during pregnancy to support healthy fetal development; (6*S*)-5-methyltetrahydrofolic acid ((6*S*)-5-MTHF) is available in some commercial prenatal vitamins as an alternative to folic acid, but its effect on blood folate status during pregnancy is unknown. To address this, we randomised sixty pregnant individuals at 8–21 weeks' gestation to 0·6 mg/d folic acid or (6*S*)-5-MTHF × 16 weeks. Fasting blood specimens were collected at baseline and after 16 weeks (endline). Erythrocyte and serum folate were quantified via microbiological assay (as globally recommended) and plasma unmetabolised folic acid (UMFA) via LC-MS/MS. Differences in biochemical folate markers between groups were explored using multivariable linear/quantile regression, adjusting for baseline concentrations, dietary folate intake and gestational weeks. At endline (*n* 54), the mean values and standard deviations (or median, inter-quartile range) of erythrocyte folate, serum folate and plasma UMFA (nmol/l) in those supplemented with (6*S*)-5-MTHF *v.* folic acid, respectively, were 1826 (SD 471) and 1998 (SD 421); 70 (SD 13) and 78 (SD 17); 0·5 (0·4, 0·8) and 1·3 (0·9, 2·1). In regression analyses, erythrocyte and serum folate did not differ by treatment group; however, concentrations of plasma UMFA in pregnancy were 0·6 nmol/l higher (95 % CI 0·2, 1·1) in those supplementing with folic acid as compared with (6*S*)-5-MTHF. In conclusion, supplementation with (6*S*)-5-MTHF may reduce plasma UMFA by ~50 % as compared with supplementation with folic acid, the biological relevance of which is unclear. As folate is currently available for purchase in both forms, the impact of circulating maternal UMFA on perinatal outcomes needs to be determined.

Key words: **Folate: Folic acid: (6*S*)-5-methyltetrahydrofolic acid: Pregnancy: Micronutrients**

Folate is an essential, water-soluble B-vitamin that is present in numerous chemically related forms (Fig. 1)[1]. Naturally occurring folates are mainly reduced and polyglutamated, with 5-methyltetrahydrofolate (5-MTHF) being the predominant form in food and systemic circulation[1,2]. Reduced folates serve as methyl donors in one carbon metabolism; thus, folate is critical during periods of growth, such as pregnancy, as it supports numerous physiological processes including cellular proliferation, re-methylation of homocysteine to methionine, nucleic acid synthesis and methylation of DNA, RNA, proteins and phospholipids[3–5].

To reduce the risk of neural tube defects (NTD), North American public health agencies advise all individuals of reproductive age to consume 0·4 mg/d folic acid, starting preconceptionally and continued throughout pregnancy and lactation[6–9]. However, prenatal vitamins in these regions generally contain 0·6–1·0 mg folic acid[10–12] and concerns have been raised regarding excessive intake in pregnancy[10,13]. Folic acid is a synthetic, oxidised form of folate (Fig. 1(a))[1,2]; as such, folic acid requires reduction by dihydrofolate reductase for use in one carbon metabolism[14]. However, unmetabolised folic acid (UMFA) is detected in biological fluids upon folic acid intake, suggesting that the capacity of intestinal dihydrofolate reductase to reduce folic acid in humans may be limited[15,16]. The biological and clinical relevance of UMFA on human health remains unclear[17].

Abbreviations: DFE, dietary folate equivalent; IQR, inter-quartile range; NTD, neural tube defect; PLP, pyridoxal phosphate; UMFA, unmetabolised folic acid; 5-MTHF, 5-methyltetrahydrofolate, (6*S*)-5-MTHF, (6*S*)-5-methyltetrahydrofolic acid.

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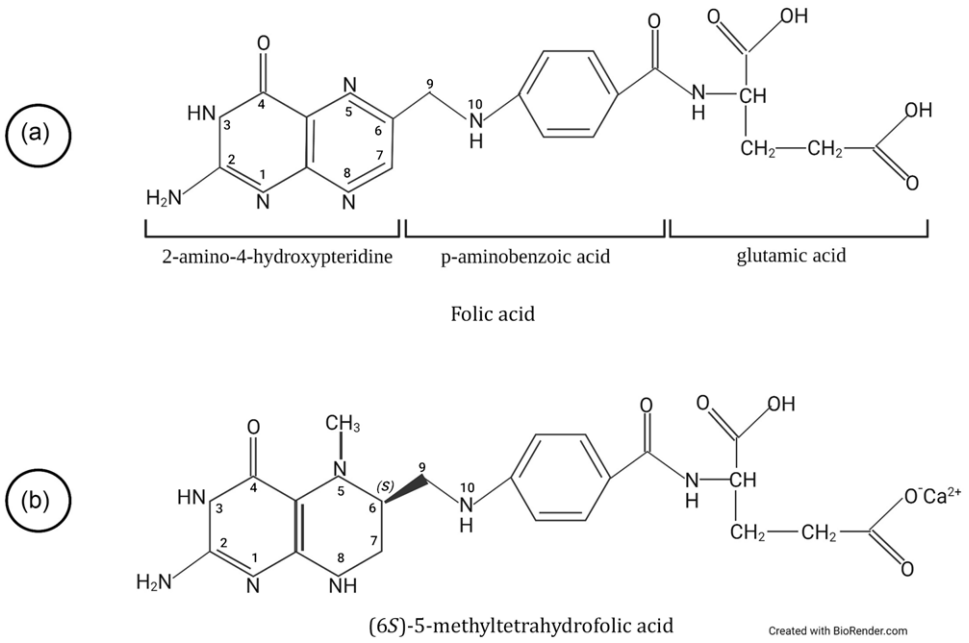

Fig. 1. Chemical structure of folic acid and (6S)-5-methyltetrahydrofolic acid. (a) Folic acid; a synthetic analogue of the folate parent structure. Folic acid is fully oxidised, increasing its stability in supplements and fortified foods, and requires reduction via dihydrofolate reductase for co-enzymatic function. (b) Ca salt of (6S)-5-methyltetrahydrofolic acid (Metafolin®); a synthetic analogue of natural 5-methyltetrahydrofolate.

An increasing number of prenatal vitamins contain (6*S*)-5-methyltetrahydrofolic acid (Fig. 1(b); (6*S*)-5-MTHF)) as an alternative to folic acid[11,18]. In 2019, 32 % of prescription and 25 % of non-prescription prenatal vitamins commercially available in the USA (as per the NIH Dietary Supplement Label and DailyMed databases) contained (6*S*)-5-MTHF; prior to 2015, none was listed[11]. Supplements in Canada which are marketed for use in pregnancy may contain folic acid or (6*S*)-5-MTHF, so long as a minimum dose of 0·4 mg is provided[18]. A possible metabolic advantage of (6*S*)-5-MTHF is that it does not require reduction via dihydrofolate reductase, as it is already in an active form[14]. However, there is no clear messaging from public health agencies regarding the difference of supplemental folate forms for pregnant individuals, as the effect of (6*S*)-5-MTHF on folate status during pregnancy has never been evaluated, and only folic acid has been shown to reduce the risk of NTD in randomised controlled trials[19–21].

While supplementation with (6*S*)-5-MTHF has proven more effective than folic acid in non-pregnant women[22–25], pregnancy is a unique physiological state associated with numerous metabolic changes, including increased haemodilution, urinary excretion and micronutrient needs to support growth of maternal and fetal tissue[26,27]. Maternal plasma supplies folate to the fetus and uteroplacental organs to accommodate growth within these compartments[26]. Placental folate receptors are established in early pregnancy[28], and folate accumulates in the intervillous space at a concentration three times that of maternal plasma for transportation of folate to the fetus against a concentration gradient[26,29]; further, in ex-vivo experiments, the transport of folate from maternal to fetal perfusate has been found to be non-saturable[26,30]. As (6*S*)-5-MTHF supplements are currently widely consumed by pregnant individuals[11], despite a lack of evidence in pregnancy, it is imperative to ensure that (6*S*)-5-MTHF can maintain folate status to the same extent as folic acid during pregnancy.

This study aimed to generate estimates of folate status, including erythrocyte folate, serum folate and plasma UMFA following supplementation with 0·6 mg/d (6*S*)-5-MTHF or folic acid × 16 weeks of pregnancy. These estimates are a critical first step in understanding any differences between commercially available folate forms.

Methods

Study participants and design

The protocol for this two-armed randomised double-blind trial has previously been published[31]. In brief, individuals aged 19–42 years with singleton pregnancies in Vancouver, Canada were recruited via printed posters and social media advertisements; full details on the recruitment methods are published elsewhere[32]. The study took place between September 2019 and September 2021. Exclusion criteria included medical conditions, medications and behavioural factors (current smoking, alcohol consumption or recreational drug use) associated with altered folate status[8], a pre-pregnancy BMI ≥ 30 kg/m^2 and being medium to high risk for development of an NTD-affected pregnancy[8]. The trial is registered at ClinicalTrials.gov (NCT04022135). The study supplements were approved for clinical trial use by the natural and non-prescription health products directorate of Health Canada (Submission No. 244456). This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the UBC Clinical Research

Ethics Board (H18-02635). Written informed consent was obtained from all subjects.

Eligible participants were scheduled for a baseline visit between 8 and 21 weeks' gestation, with instructions to continue any folate supplementation until the day before this visit. At baseline, written informed consent was obtained (including confirmation of eligibility via a checklist), weight and height were measured and demographic information and usual intake of dietary folate equivalents (DFE; as per a validated FFQ; NutritionQuest[33]) were collected. The Block Folic Acid/DFE Screener was used; this FFQ includes twenty-one items identified as the top dietary folate contributors as per National Health and nutrition examination survey (NHANES) 1999–2000. DFE are calculated based on the respondent's reported frequency of consumption of each item, a default portion size (per the respondent's age and sex) and the folate content listed in the US Department of Agriculture Food and Nutrient Database for Dietary Studies[33]. A conversion factor of 1·7 is used to convert µg supplemental folate (from supplements and folic acid fortified foods) to 1 µg DFE; 1 µg folate from natural food sources is equivalent to 1 µg DFE[33,34].

Participants were randomised to 0·6 mg/d folic acid or an equimolar dose (0·625 mg/d) of Metafolin® (Ca salt of (6*S*)-5-MTHF), based on molecular weights of 441·4 and 459·5 g/mol for folic acid and Metafolin®, respectively; a prenatal vitamin (NPN: 80025456) with folic acid removed was also provided to all participants. The randomisation sequence was produced by an independent statistician using permuted, equal blocks of four. Blinded allocations ('A' or 'B') for each study ID were kept in individual envelopes prepared by a research volunteer. Participants were assigned a unique study ID, and at baseline visits, the allocated supplements for that study ID were provided. Participants and the research team remained blinded to folate group allocations until final statistical analyses were complete. Participants were given all supplements needed for the 16-week intervention period and were instructed to begin supplementation immediately following baseline visits. Participants were contacted at the study midpoint (8 weeks after the baseline visit) to touch base and answer any questions about the trial.

Following 16 weeks, participants were scheduled for a second visit (endline). Participants had the option to continue supplementation for the postpartum study phase, to explore the effect on folate status after delivery (~1 week postpartum); new written informed consent was obtained, and more study supplements were provided to those interested. Capsule counts were completed at endline and postpartum visits to assess adherence to daily supplementation. Participants were also given a supplement diary to record daily intake or reasons for missing a dose.

Blood collection and processing

Venous blood specimens were collected at baseline, endline and postpartum visits in a 4 ml serum tube, 6 ml EDTA tube and 2 ml EDTA tube (Becton Dickinson). At baseline and endline visits, participants were instructed to fast for 3 h to mitigate the influence of recent folic acid intake on plasma UMFA[16,35]. Blood specimen collections at postpartum visits were non-fasting to reduce participant burden, with instruction to consume the folate supplement 2 h prior to the visit (to standardise any peak in plasma UMFA following intake); erythrocyte folate is less sensitive to recent intake[34]. After collection, tubes were shielded from light, inverted gently and transported to the laboratory for immediate processing (within 2 h of collection).

Whole blood (0·3 ml) was removed from the 6 ml EDTA tube and was diluted 1/11 with a 3 ml 1 % ascorbic acid solution, followed by incubation at 37°C for 30 min[36]. Remaining whole blood in the 6 ml EDTA tube was centrifuged at 3000 rpm for 15 min at 4°C; plasma was separated into aliquots. Remaining contents in the 6 ml EDTA tube were processed for peripheral blood mononuclear cell isolation (SepMate™ STEMCELL Technologies). The 2 ml EDTA tube was used for a complete blood count, including haematocrit (L/L) determination via an automated haematology analyser (Sysmex XNL-550, Sysmex Corp.). Serum tubes were left at room temperature to clot for minimally 30 min and then centrifuged at 3000 rpm for 10 min at 4°C; serum was separated into aliquots. All specimens were frozen immediately after processing at –80°C until analysis.

Biochemical analyses

Total serum and whole blood folate (nmol/l) were determined as per the microbiological assay (Bevital AS) with chloramphenicol-resistant *Lactobacillus rhamnosus* and folic acid calibration, using previously described methods[37]. The inter-assay CV ranged from 3·1 to 5·6 % based on three quality controls that were analysed in duplicate. Erythrocyte folate (nmol/l) was subsequently calculated as recommended[36]:

$$\text{Erythrocyte folate} = \frac{(\textit{Whole blood haemolysate folate} \times 11) - \textit{Serum folate}\,(1 - \textit{Haematocrit}/100)}{\textit{Haematocrit}/100}$$

Plasma UMFA (nmol/l) was determined via LC-MS/MS as previously described[38–42]. The inter-assay CV for plasma UMFA was 8 %, as determined using internal quality control samples. Exploratory outcomes included other methyl nutrients and *MTHFR* 677 C > T variant genotyping. Plasma vitamin B_{12} (pmol/l) was determined via an immunoanalyser (Abbott Architect i1000; CV = 3·3 %). Plasma pyridoxal phosphate (PLP; nmol/l; CV = 8 %), total homocysteine (µmol/l; CV = 1·7 %), cysteine (µmol/l; CV = 1·4 %), methionine (µmol/l; CV = 1·1 %), free choline (µmol/l; CV = 5·4 %) and betaine (µmol/l; CV = 2·7 %) were determined using high-performance LC-MS/MS[43,44]. Genotyping of *MTHFR* 677 C > T variant (*rs*1801133) was determined via pyrosequencing using Pyromark™ Q96 MD Pyrosequencer (Qiagen), as previously described[45]. Plasma vitamin B_{12}, PLP, total homocysteine, cysteine, methionine, free choline and betaine were quantified at baseline and endline visits only.

Statistical analyses

Participant demographic data and biochemical outcomes were summarised descriptively. Folate status in each group (erythrocyte folate, serum folate and plasma UMFA) was summarised using mean values and standard deviations or medians with

inter-quartile ranges (IQR) if not normally distributed. The median (IQR) within-person change in folate status (erythrocyte folate, serum folate and plasma UMFA) from baseline to endline was calculated. The change in concentrations of erythrocyte folate, serum folate and plasma UMFA in each intervention group was further explored by evaluating differences in endline concentrations, adjusting for baseline concentrations, dietary folate intake and weeks gestation at supplement initiation, using multivariable linear regression (or multivariable quantile regression for non-normally distributed outcomes). Quantile regression was chosen over data transformations as it requires fewer assumptions about the underlying distribution and leads to more straightforward biological interpretations of the model estimates[46]. Differences in postpartum erythrocyte folate were explored using quantile regression, adjusting for endline concentrations, dietary folate intake and total weeks supplementing. Postpartum concentrations of serum folate and plasma UMFA were not explored in adjusted models given their limited interpretability due to the non-fasting state. Covariates were chosen a priori; however, in a post-hoc protocol change, we opted not to adjust for exploratory biomarkers (other methyl nutrients, *MTHFR* genotype) as no clinically meaningful differences in biochemical markers between groups were observed, and due to few participants with the homozygous (TT) polymorphism (n 2 in the folic acid group). All analyses were completed on an intention-to-treat basis. Due to high adherence to the study protocol throughout the trial (median (IQR) adherence to daily supplementation = 98 % (96 %, 100 %), as per capsule counts), per-protocol analyses were deemed unnecessary. A P value < 0·05 was considered statistically significant.

Sample size considerations

The sample size was based on the number of participants generally recognised as sufficient for conducting a pilot study in clinical research (n 50)[47], with the aim of generating estimates that can be used to inform a definitive trial. To account for an estimated 20 % attrition, we aimed to recruit a total of sixty participants to the current study.

Results

Overall, sixty participants completed baseline visits, fifty-four completed endline visits (retention rate of 90 %) and thirty-seven provided a postpartum blood specimen (retention rate of 69 %). One participant delivered prematurely and could not participate in the endline visit; however, they provided a postpartum blood specimen. See Fig. 2 for the participant flow diagram. Adherence to daily supplementation as per capsule counts was high, with rates of daily supplementation in 93 % (n 50) of participants ≥ 90 %. At postpartum visits, 92 % (n 34) of participants had rates of daily adherence ≥ 90 %. Per the supplement diary, primary reasons for a missed dose included: fatigue, forgetting, illness (gastrointestinal distress), being on vacation and lack of intake while admitted to hospital for delivery.

Participant characteristics are presented in Table 1. Participants appeared similar between groups and were ~33 years of age, highly educated (n 58 (97 %) with post-secondary education) and predominantly nulliparous (73 %). All participants reported a pre-pregnancy BMI < 30 km/m^2; median (IQR) gestational weight gain from baseline to endline was 0·44 kg/week (0·36–0·56) and 0·46 kg/week (0·39–0·56) in the (6*S*)-5-MTHF and folic acid groups, respectively (missing n 3 endline weights). The intervention period from baseline to endline was 16 weeks (± 6 days); however, due to COVID-19 pandemic countermeasures, endline visits for n 5 (n 2 in the (6*S*)-5-MTHF group and n 3 in the folic acid group) were moved earlier or delayed; the min/max intervention period was 13/17 weeks in the (6*S*)-5-MTHF group and 14/20 weeks in the folic acid group. The postpartum visit was planned for ~1 week after delivery; however, this varied slightly based on participant availability; the median (IQR) days postpartum in the (6*S*)-5-MTHF and folic acid groups, respectively, was day 9 (7–11 d) and day 8 (7–10 d); overall, weeks of supplementing from baseline to postpartum were 24 (SD 4) weeks.

Baseline biochemical outcomes are presented in Table 2; outcomes at endline and postpartum visits are presented in Table 3 (erythrocyte folate, serum folate and plasma UMFA) and online Supplementary Table 1 (exploratory outcomes: vitamin B_{12}, PLP, total homocysteine, cysteine, methionine, free choline and betaine). While cut-offs during pregnancy are not established, median concentrations of vitamin B_{12} and PLP remained above cut-offs for deficiency in non-pregnant women (vitamin B_{12} < 148 pmol/l and PLP < 20 nmol/l), and total homocysteine remained < 13 μmol/l (cut-off for hyperhomocysteinaemia) in all participants, at all visits[48,49]. No participants had erythrocyte folate (< 305 nmol/l) or serum folate (< 7 nmol/l) indicative of deficiency at any point[50]. Further, erythrocyte folate remained > 906 nmol/l in all participants throughout the study (a cut-off associated with maximal risk reduction of NTD[10,51]). Plasma UMFA was detectable in all participants at all timepoints.

We note a high degree of inter-individual variability of within-person change in folate status during pregnancy; the median (IQR) within-person change from baseline to endline in erythrocyte and serum folate, respectively, in those supplemented with (6*S*)-5-MTHF was 88 nmol/l (26, 346) and 2·5 nmol/l (–9·8, 15), and in those supplemented with folic acid was 368 nmol/l (–81, 549) and 0·8 nmol/l (–15, 15). In those supplemented with (6*S*)-5-MTHF, plasma UMFA decreased by a median (IQR) of 0·5 nmol/l (0·9, 0·2), representing a ~50 % decrease at endline. Conversely, in those supplemented with folic acid, plasma UMFA remained mostly consistent from baseline to endline (median (IQR) within-person change = 0·03 nmol/l (–0·2, 0·9)).

Crude and adjusted differences between intervention groups in erythrocyte folate, serum folate and plasma UMFA at endline and postpartum visits are presented in Table 3 (see online Supplementary Table 2 for full model outputs). Concentrations of erythrocyte and serum folate at endline and postpartum were not clinically different between intervention groups, as both groups were well-above cut-offs for deficiency or NTD risk reduction, with a high degree of overlap and variability in crude concentrations (± ~450 nmol/l for erythrocyte folate and ± ~15 nmol/l for serum folate, at endline); differences in erythrocyte

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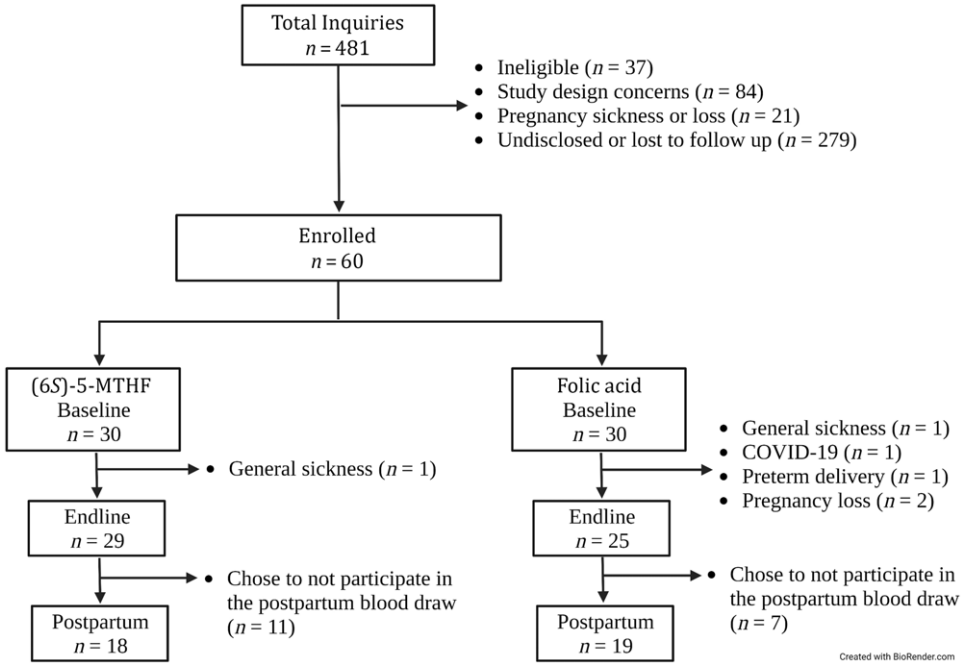

Fig. 2. Participant flow diagram. The folic acid group includes *n* 19 at the postpartum visit; this includes *n* 18 from endline (*n* 7 chose to not participate in the postpartum blood draw) and *n* 1 who delivered prematurely (thus, did not participate in the endline visit), but provided a postpartum blood specimen.

and serum folate were also not statistically significant, as the 95 % CI for all crude and adjusted differences crossed 0. Conversely, there appeared to be a meaningful difference in plasma UMFA at endline and postpartum, as there was no overlap in the IQR of crude estimates, and concentrations were significantly higher at endline (adjusted difference = 0·6 nmol/l, 95 % CI 0·2, 1·1) and postpartum (crude difference = 12 nmol/l, 95 % CI 6·1, 19) in those supplemented with folic acid as compared with (6*S*)-5-MTHF.

Discussion

In this investigation of sixty folate-replete, low-risk, singleton pregnancies, supplementation with 0·625 mg/d (6*S*)-5-MTHF and an equimolar dose of folic acid (0·6 mg/d) appeared similarly effective in maintaining folate status (erythrocyte and serum folate) in pregnancy and after delivery. The most meaningful difference between supplemental folate forms was the effect on plasma UMFA, as supplementation with folic acid resulted in significantly higher circulating concentrations of UMFA as compared with (6*S*)-5-MTHF.

Investigations in non-pregnant[22–24] and lactating[25] women have reported that (6*S*)-5-MTHF is more effective than folic acid in raising erythrocyte and/or serum folate concentrations. Bailey *et al.*[22] conducted a pharmacokinetic investigation of 7·5 mg/d (6*S*)-5-MTHF or folic acid × 3 d, followed by 0·4 mg/d × 2 weeks; those supplemented with (6*S*)-5-MTHF more quickly achieved the target cut-off (serum total folate >50 nmol/l), but by day 12, there were no differences between groups[22]. Henderson *et al.*[23] and Lamers *et al.*[24] investigated (6*S*)-5-MTHF and folic acid supplementation at 1·0 mg/d × 12 weeks[23] and 0·4 mg/d × 24 weeks[24] in non-pregnant women of childbearing age; both reported greater increases in erythrocyte folate concentrations following (6*S*)-5-MTHF supplementation and an equal or increased effect on plasma folate concentrations. Similarly, Houghton *et al.*[25] reported significantly higher erythrocyte folate concentrations in those supplementing with (6*S*)-5-MTHF *v.* folic acid at 0·4 mg/d × 16 weeks during lactation. While we did not investigate pharmacokinetic action, we propose that the lack of differences in erythrocyte and serum folate found between groups in the current study may be attributed to changes in folate handling during pregnancy[26]; perhaps any increased effect of (6*S*)-5-MTHF on folate status as compared with folic acid was nullified due to the abundant transfer of folate from maternal plasma to the placenta and fetus, or due to increased folate catabolism and excretion, particularly in later pregnancy[52,53].

While the difference in plasma concentrations of UMFA between groups was modest after fasting, there did appear to be a meaningful effect driven by supplemental folate form. All participants reported supplementation with folate prior to study enrolment; as per the type of folate or brand of prenatal vitamin, >90 % consumed folic acid. At baseline visits, plasma UMFA was ~1 nmol/l in both groups; we did not observe continued increases of UMFA in the folic acid group (UMFA remained at ~1 nmol/l at endline); however, concentrations in the (6*S*)-5-MTHF group decreased by ~50 %. Fasting state and folic acid supplementation have previously been identified as the strongest predictors of circulating UMFA[54,55]. We do suspect a high degree of UMFA clearance following the (minimum) 3 h fast, given comparison with non-fasting postpartum UMFA values (see Table 3). However, as per NHANES 2007–2008[55], plasma UMFA concentrations remain significantly higher after

Table 1. Participant characteristics of pregnant individuals supplemented with (6*S*)-5-MTHF or folic acid (Vancouver, Canada, 2019–2021) (Numbers and percentages; mean values and standard deviations; medians and inter-quartile ranges)

	(6*S*)-5-MTHF (*n* 30)		Folic acid (*n* 30)	
	n	%	*n*	%
Age, years				
Mean	33		33	
SD	3·1		3·8	
Ethnicity				
European	16	53	18	60
South, East and Southeast Asian	6	20	7	23
Hispanic/Latino	5	17	2	7
Middle Eastern	2	7	0	0
Mixed ethnicity	1	3	3	10
Education				
High school	1	3	1	3
College	7	23	2	7
Undergraduate	10	34	15	50
Graduate	12	40	12	40
Household income per year, CAD$*				
< 20 000	1	3	0	0
20 000–50 000	2	7	4	14
50 000–100 000	10	34	11	39
> 100 000	17	56	13	47
Nulliparous	24	80	20	67
	Median	IQR	Median	IQR
Dietary folate intake, mg DFE/day†				
From foods	0·53	0·17	0·45	0·16
From supplements‡	0·7	0·7, 0·7	0·7	0·7, 0·7
Total intake	1·2	1·1, 1·4	1·2	1, 1·3
Special diet followed§				
n	4		4	
%	13		13	
Weeks gestation	18	15, 20	17	13, 20
Min/max weeks	9/21		8/21	

(6*S*)-5-MTHF, (6*S*)-5-methyltetrahydrofolic acid; DFE, dietary folate equivalents.
* Missing *n* 2 household income in the folic acid group (participants chose not to disclose).
† As determined by the FFQ.
‡ Supplementation prior to study enrolment; all participants reported folate supplementation prior to study enrolment, which was discontinued and replaced with the study vitamins at baseline.
§ Lacto-ovo-vegetarian (*n* 1), pescatarian (*n* 1), vegan (*n* 1), gluten-free (*n* 3), other (*n* 2).

Table 2. Baseline biochemical outcomes in pregnant individuals supplemented with (*6S*)-5-MTHF or folic acid (Vancouver, Canada, 2019–2021) (Medians and inter-quartile ranges; numbers and percentages)

	(6*S*)-5-MTHF (*n* 30)		Folic acid (*n* 30)	
	Median	IQR	Median	IQR
Erythrocyte folate (nmol/l)	1574	1380, 1811	1656	1342, 1957
Serum folate (nmol/l)	65	60, 73	68	61, 85
Plasma UMFA (nmol/l)	1·2	0·8, 1·5	1·1	0·9, 1·5
Vitamin B_{12} (pmol/l)	288	210, 349	299	222, 349
Pyridoxal phosphate (nmol/l)	55	38, 94	88	42, 101
Total homocysteine (μmol/l)	4·7	4, 4·9	5	4·5, 5·6
Cysteine (μmol/l)	236	217, 254	256	233, 271
Methionine (μmol/l)	25	24, 29	25	23, 27
Free choline (μmol/l)	7·2	5·9, 8·2	7·1	6·1, 8·1
Betaine (μmol/l)	15	14, 18	16	13, 20
	n	%	*n*	%
MTHFR 677 C > T genotype				
CC	16	53	16	53
CT	8	27	12	40
TT	6	20	2	7

(6*S*)-5-MTHF, (6*S*)-5-methyltetrahydrofolic acid; UMFA, unmetabolised folic acid.
Results are median (IQR) unless otherwise noted. Missing *n* 1 in the (6*S*)-5-MTHF group for the assessment of some exploratory plasma outcomes due to insufficient blood sample (*n* 29 for pyridoxal phosphate, total homocysteine, cysteine, methionine, choline, betaine).

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Table 3. Effect of supplemental folate form on late pregnancy and postpartum erythrocyte folate, serum folate and plasma UMFA among pregnant individuals supplemented with (6*S*)-5-MTHF or folic acid (Vancouver, Canada, 2019–2021) (Mean values and standard deviation; 95 % confidence intervals)

	(6*S*)-5-MTHF		Folic acid					
	Crude concentrations		Crude concentrations					
	Mean	SD	Mean	SD	Crude difference	95 % CI	Adjusted difference*	95 % CI
Erythrocyte folate (nmol/l)								
Endline	1826	471	1998	421	171	–74, 417	161	–79, 400
Postpartum								
Median	1724		1962		239	–159, 636	11	–272, 294
IQR	1474, 1877		1576, 2284					
Serum folate (nmol/l)								
Endline	70	13	78	17	7·7	–0·6, 16	7·4	–1·4, 16
Postpartum	96	39	104	34	8·9	–16, 33	–	
Plasma UMFA (nmol/l)								
Endline								
Median	0·5		1·3		0·7	0·3, 1·1	0·6	0·2, 1·1
IQR	0·4, 0·8		0·9, 2·1					
Postpartum								
Median	0·8		13		12	6·1, 19	–	
IQR	0·7, 1·2		7, 23					

UMFA, unmetabolised folic acid; (6*S*)-5-MTHF, (6*S*)-5-methyltetrahydrofolic acid; IQR, inter-quartile range.
n 54 participants included for all endline analyses (crude and adjusted); *n* 36 participants included for crude estimates of postpartum erythrocyte folate and serum folate (missing *n* 1 due to insufficient blood sample); *n* 35 participants included for adjusted estimates of postpartum erythrocyte folate (missing *n* 2 due to (1) insufficient blood sample and (2) missed endline visit due to premature delivery, participated in postpartum visit only); *n* 37 participants included for crude estimates of postpartum plasma UMFA.
* Endline outcomes adjusted for: folate form ((6*S*)-5-MTHF as the reference group), baseline values (nmol/l), weeks of gestation at baseline and dietary folate intake (mg dietary folate equivalents/d). Postpartum erythrocyte folate adjusted for: folate form ((6*S*)-5-MTHF as the reference group), endline values (nmol/l), total weeks supplementing and dietary folate intake (mg dietary folate equivalents/d).

fasting ≥ 8 h in folic acid supplement users (~1 nmol/l) *v.* non-supplement users (~0·7 nmol/l). Overall, it seems that a significant reduction of UMFA in plasma can be effectively achieved by supplementation with (6*S*)-5-MTHF. Ultimately, it is noted that while plasma UMFA is different between groups, after fasting, concentrations are relatively low overall. Thus, the biological relevance of this difference is uncertain.

Excess folic acid intake has been associated with various adverse health outcomes for both the mother and child[10,13,17]. Clinical concerns related to offspring health include neuro-developmental disorders[56–63], allergic diseases[64–68], metabolic outcomes[17,69–72] and poor fetal growth (small-for-gestational-age birth)[73,74]. However, results in human studies are mixed, with some reporting a harmful association[58,63–65,73], null findings[56,59,66–68,75] or a protective effect[57,60–62,74] of maternal folic acid supplementation. Further, the effects of folic acid may vary by dose; for example, folic acid supplementation at recommended doses (0·4 mg/d) is associated with a reduced risk of small-for-gestational-age[74], whereas intakes > 1·0 mg have shown an increased risk[73]. It is proposed that UMFA demonstrates a dose–response relation with adverse outcomes, whereby higher UMFA is associated with greater risk[13,76].

Interpretation of findings is further complicated by a lack of clarity as to whether concerns are related specifically to folic acid, or more generally, a 'high' folate status. While a cut-off for 'high' erythrocyte folate is not established, 1360 nmol/l was reflective of the 97th percentile as per NHANES 1999–2004[77,78]. Overall, it is very difficult for pregnant individuals in North America to consume only 0·4 mg/d folic acid, due to folic acid food fortification and as the vast majority of prenatal vitamins contain 0·6–1·0 mg folic acid[10,11,79–81]; this has contributed to very high folate levels (erythrocyte folate ~1500–2500 nmol/l) among pregnant and lactating individuals[10,82–84]; similarly high erythrocyte and serum folate concentrations were evident in the current study. Those consuming folic acid from fortified foods and prenatal vitamins are very likely to exceed the tolerable upper intake level of 1·0 mg/d folic acid[12,79–81,85].

Other considerations regarding the use of (6*S*)-5-MTHF *v.* folic acid include the lack of an established conversion factor of (6*S*)-5-MTHF to DFE, stability differences and cost. North American supplement manufacturers are permitted to use the folic acid factor of 1·7 to convert µg (6*S*)-5-MTHF to DFE[11,17]. Appropriateness of this is unclear, given potential differences in bioavailability as observed in non-pregnant populations[86]; however, findings of the current trial suggest that bioavailability of folate forms during pregnancy may differ from non-pregnant individuals, in that a greater effect of (6*S*)-5-MTHF on folate status was not observed at a 0·6 mg dose. Ca salt of (6*S*)-5-MTHF (Metafolin®) is stable in its crystalline form for use in supplements[87,88]. As a food fortificant in white flour, stability of (6*S*)-5-MTHF can be improved via microencapsulation technology, alone or in combination with sodium ascorbate[89,90]; however, large-scale implementation of these techniques has had limited success in practice[91] and only folic acid is currently used in North American food fortification programmes[92]. Finally, given the increased cost of (6*S*)-5-MTHF *v.* folic acid, compelling evidence of its improved safety as compared with folic acid is likely required before it would be considered for use in population-wide food fortification programmes.

Ultimately, a definitive trial is required to confirm that erythrocyte and serum folate are similarly maintained following supplementation with (6*S*)-5-MTHF and folic acid during

pregnancy; while we found no evidence for a clinically important difference, the upper end of the 95 % CI for the adjusted difference in erythrocyte folate between groups at endline was 400 nmol/l, favouring a stronger response following folic acid supplementation. Interpretation of this is limited as a non-inferiority margin was not pre-specified in our protocol, as this was a pilot trial. Overall, we hypothesise that the 95 % CI would tighten following assessment in a larger sample size, suggesting a similar effect of both forms at a 0·6 mg dose, given that overall there was a high degree of variability in folate estimates, 95 % CIs were wide and crossed 0, and a stronger response to folic acid contradicts the body of evidence on dose–response of folic acid *v.* (6*S*)-5-MTHF[86]. Both biochemical (e.g. folate status, plasma UMFA) and clinical outcomes should be evaluated in a definitive trial; we suggest assessment of fetal growth and neurodevelopment as outcomes of interest[10,13].

Limitations include the small sample size, as this was a pilot trial which aimed to generate estimates for a definitive trial. While a 3 h fast was selected to mitigate the effect of folic acid on plasma UMFA[16,35], total serum folate concentrations may still have been influenced by recent intake within this time frame[34,55]. Estimation of dietary folate intake was only assessed once (at baseline); thus, its accuracy in later pregnancy and after delivery is uncertain. Dietary folate assessment via an FFQ can be useful when assessing a single nutrient over a longer period, as it captures food sources that may be consumed only occasionally and thus may be missed via other methods[93]. However, previous research indicates that FFQ may overestimate folate intake[93,94]. We further note that the FFQ used was an American tool; while dietary patterns of those in Canada *v.* the USA may differ, green salads, orange juice, bread products and cold cereals are reported as top folate contributors for pregnant and lactating Canadians[80,95], all of which were included on the folate screener. Both countries fortify foods with folic acid at similar rates (0·14 and 0·15 mg per 100 g white flour and cereals in the USA and Canada, respectively), aiming to provide ~0·1 mg/d[96]. However, DFE of folate supplementation prior to the trial as per the FFQ are likely underestimated; the median (IQR) mg DFE from supplements was 0·7 (0·7, 0·7 mg DFE), assuming a folic acid dose of 0·4 mg (×1·7 = 0·7 mg DFE). While the dose of folate provided in the study (0·6 mg/d) was chosen to improve generalisability, given that it matches folic acid content in leading Canadian brands (Materna®), over-the-counter prenatal vitamins contain varying quantities of folate (often 1 mg in Canada). Finally, timing of supplement initiation varied widely (8–21 weeks). While folate recommendations in Canada and the USA do not differ based on gestational weeks, we note a potential limitation when comparing folate status of those across different stages of pregnancy.

In conclusion, supplementation with (6*S*)-5-MTHF resulted in similar erythrocyte and serum folate concentrations as folic acid during pregnancy and after delivery, while reducing maternal plasma UMFA. More research is needed to confirm whether there is any risk associated with folic acid supplementation in pregnancy (particularly at doses > 0·4 mg) or the presence of plasma UMFA. This should be followed by the establishment of high-risk cut-offs for maternal folate status, which can be incorporated into perinatal clinical practice guidelines, and re-formulation of prenatal vitamins available in North America (perhaps by lowering the dose to 0·4 mg), to support achievement of optimal maternal folate status. Results of this study should be confirmed in a definitive trial; however, the current findings are very timely and of interest immediately, as both folate forms are currently widely available and consumed in pregnancy at a dose of 0·6 mg/d or greater.

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Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114523001733

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