## Background

**Folate metabolism.** Folate is a B-vitamin that plays a critical role in cell growth and division, erythropoiesis, and DNA synthesis, repair, and methylation.[1] Folate from natural sources in food is in the reduced form and enters circulation directly as 5-methyltetrahydrofolate (5-MTHF) (**Figure 1 in the Appendix**). Folic acid is the synthetic form of folate that is used in supplements and food fortification (e.g. fortified flour) due to its high stability and bioavailability. Folic acid is different from naturally occurring folate, as it is fully oxidized, has a different biochemical structure, and is metabolized differently.[2] Folic acid must first be reduced to dihydrofolate (DHF) by an enzyme called dihydrofolate reductase (DHFR). It is then reduced further in a series of steps to 5-MTHF, before entering circulation.[3]

**Folate in early pregnancy is critical for preventing neural tube defects (NTDs).** Neural tube closure is an early developmental event that fuses the cranium and spinal cord.[4] Full closure occurs between 21 to 28 days post-conception. Failure of proper neural tube closure can cause death soon after birth or lead to serious long-term disability. Folate taken before and during early pregnancy reduces the risk of an NTD. In a multi-center RCT, folic acid supplementation reduced NTD recurrence by 72% (95% CI: 29 to 92%) in babies whose mother had a prior history of NTD.[5] Several randomized trials[6] and a large non-randomized trial in China ($n$=250,000)[7] demonstrated significant protection against NTD in women without a prior history of an NTD-affected pregnancy with doses of folic acid as low as 0.4 mg/d. As a result, the Society of Obstetrics and Gynecologists of Canada recommend that all women of childbearing age take between 0.4-4 mg/d folic acid, depending on NTD history and calculated risk level, starting 2-3 months before conception.[8]

**Most babies in BC are exposed to synthetic folic acid supplements in utero.** Reports indicate that 94% of pregnant women in BC took a multivitamin containing folic acid during the first three months of pregnancy.[9] Of these, 92% reported taking it daily.[9] Thus, the majority of babies in BC are likely exposed to doses of folic acid of >0.4 mg/d, as most prenatal multivitamin supplements contain higher doses[8] (e.g. Materna® contains 0.6 mg folic acid[10]).

**Too much folic acid may be harmful.** The capacity of the DHFR enzyme to convert synthetic folic acid to DHF is limited[11] and the gut cannot metabolize more than ~0.2 mg folic acid at a time.[12,13] With doses as low as 0.2 mg/d in healthy individuals, unmetabolized folic acid has beed detected in circulation (**Figure 1 in the Appendix**).[14] The body's inability to fully metabolize synthetic folic acid is a concern, because high synthetic folic acid intakes have been associated with acceleration of some cancers, including mammary tumors in rats,[15] colorectal cancer in men and women,[16] prostate cancer in men,[17] and breast cancer in women.[18] Further, excess synthetic folic acid may interfere with folate metabolism and DNA methylation.[19,20] For example, high synthetic folic acid intakes (resulting in high levels of DHF) could potentially inhibit the formation of 5-MTHF, leading to a decreased synthesis of other key methyl donors, methionine and *S*-adenosyl-methionine (SAM).[21] Imbalances in folate metabolism (e.g. altered levels of key methyl donors) could affect DNA methylation and gene expression.[22] This is especially concerning in pregnancy as the baby is undergoing rapid growth and development.

**There is mounting concern that Canadian babies are exposed to too much folic acid in utero.** A recent study of 368 Canadian mothers who consumed perinatal folic acid (between ~0.4 to 1 mg/d) showed that plasma unmetabolized folic acid was detectable in >90% of maternal and newborn cord blood samples.[23] Authors concluded that doses of folic acid >0.4 mg/d likely exceeded the physiologic capacity to metabolize folic acid and should be reconsidered, especially among those consuming folic acid fortified foods.[24] This raises serious concern regarding the policy of perinatal folic acid supplementation in Canada.

**A natural form of folate may be safer than synthetic folic acid.** A naturally-occurring reduced form of folate, (6S)-5-MTHF (Metafolin®), is commercially available in nutrition supplements including prenatal multivitamins.[25] This form does not require enzymatic activation as it can directly enter circulation. Supplementation with (6S)-5-MTHF has shown to increase blood folate in healthy men and women,[26–28] and lactating women,[29] but has yet to be evaluated in pregnancy. Physiological changes during pregnancy, such as hemodilution, altered renal function, or increased inflammation, may affect how folic acid is metabolized in pregnancy. This may consequently influence blood folate concentrations. It is critical to ensure that natural folate can maintain serum folate concentrations during pregnancy, as it is the serum that supplies the folate to the growing fetus. (6S)-5-MTHF is also thought to be safer than folic acid, as it does not produce unmetabolized folic acid, as do doses of folic acid ≥0.2 mg/d. Thus, a proof-of-concept trial is needed to assess the effectiveness and safety of (6S)-5-MTHF, as compared to folic acid, during pregnancy. Given the lack of evidence on the effect of this natural folate during pregnancy, we propose to first conduct a trial during pregnancy after neural tube closure (to reduce the risk of harm should it prove less effective) to generate pilot data to inform a definitive trial.

## RESEARCH PLAN

Our **overarching goal** is to determine if the natural (6S)-5-MTHF is as effective as folic acid in increasing serum and RBC folate concentrations during pregnancy, while resulting in lower plasma unmetabolized folic acid levels, as compared to folic acid. We **hypothesize** that supplementation with (6S)-5-MTHF will result in comparable levels of serum and RBC folate, and lower levels blood unmetabolized folic acid, as compared to supplementation with folic acid. A randomized non-inferiority trial is needed to achieve this overarching goal. In order to inform the design of this definitive trial, a pilot study of (6S)-5-MTHF during pregnancy is critical.

**The specific aims of this pilot study are:**

1) To establish the mean ± standard deviation change in serum folate, RBC folate, and unmetabolized folic acid in each group following supplementation with (6S)-5-MTHF or folic acid
2) To determine participation recruitment and retention rate, the most effective recruitment strategies for this population, and adherence to study protocol (to inform a definitive trial)

**Study design:** A pilot study of supplementation of 0.6 mg/d folic acid (the same dose as in Materna®) or an equimolar dose (0.625 mg/d) (6S)-5-MTHF in pregnant women for ~16 weeks of their pregnancy

(beginning after the neural tube closure). Both groups will also receive a prenatal multivitamin (which will not contain any additional folic acid/folate), to ensure nutritional adequacy of other micronutrients important during pregnancy (e.g. iron, calcium, etc.). See study supplement nutrition facts outlined in **Table 1** (Appendix). This will also inform women's acceptability to consume a multivitamin during pregnancy for the future trial. A supplement diary will be provided to participants at the baseline meeting and will include a calendar to easily record daily supplement intake, any missed supplements/the reason for missing a daily dose, and any perceived side effects. This supplement diary and any leftover pills will be given back at the endline meeting in order to assess participant adherence to the study protocol.

**Study visits:** are outlined in **Table 2** (Appendix). A total of two visits will be required (a total of approximately 1.5 hours). Enrolled women will be asked to continue any folic acid or other micronutrient supplements, as they were previously taking, until their baseline visit, which will take place at 8-21 weeks gestation. At the baseline visit, a blood sample will be collected and women will be randomized to a folate group (0.6 mg folic acid or 0.625 mg (6S)-5-MTHF). Following 16 weeks of supplementation , an endline blood sample will be collected.

**Population and recruitment:** Pregnant women living in the greater Vancouver area (aged 19-42 y) will be recruited mainly from a variety of medical clinics, prenatal clinics, and midwife clinics throughout the city. Ms. Cochrane will call clinics to provide information on the trial, and discuss recruitment strategy options, including:

1) Hanging recruitment poster(s) throughout the clinic (e.g. in waiting rooms, patient rooms, etc)

2) Having Ms. Cochrane (graduate student/research coordinator) present study information to clinic health care professionals (e.g. physicians, nurses, medical office assistants, etc.).

We will also hang the recruitment poster throughout Vancouver in establishments visited frequently by pregnant women (e.g. prenatal classes, prenatal fitness/yoga studios, maternity clothing stores, etc), and post it electronically on social media sites viewed by pregnant women in Vancouver (e.g. prenatal groups on Facebook).

Ms. Cochrane will present study details at the Vancouver midwife and obgyn monthly meetings; we will provide posters for them to display in their clinics.

**Inclusion criteria**: i) pregnant woman (singleton pregnancy); ii) living in greater Vancouver area and willing to travel to the University of British Columbia for study visits; iii) <21 weeks gestation at time of consent; iv) 19-42 years; v) willing to participate

**Exclusion criteria:** i) having a pre-existing medical condition known to impact maternal folate status (malabsorptive and inflammatory bowel diseases, active celiac disease, gastric bypass surgery, atrophic gastritis, epilepsy, advanced liver disease, kidney dialysis, type 1 or 2 diabetes mellitus, sickle cell trait/anemia); ii) lifestyle factors known to impact maternal folate status (current smoking, alcohol consumption, recreational drug use); iii) are medium to high risk for development of an NTD pregnancy (applies to women or their male partner: personal or family history [parents or siblings] of

other folate sensitive congenital anomalies, personal NTD history or a previous NTD pregnancy); iv) are taking medications known to interfere with B-vitamin metabolism (Chloramphenicol, Methotrexate, Metformin, Sulfasalazine, Phenobarbital, Phenytoin, Primidone, Triamterene, Barbiturates); v) pre-pregnancy body mass index (BMI) $\geq$30kg/m$^2$; or vi) allergy to any study supplement ingredients

We require 50 women (25 in each group) to reliably estimate the distributions of serum and RBC folate. Thus, to account for drop outs or loss to follow up, we will recruit a total of 60 women (30 in each group).

**Study Supplements:** Bulk ingredients for the folic acid and prenatal multivitamins have been provided by Natural Factors (Coquitlam, Canada). Bulk (6S)-5-MTHF (Metafolin®) has been provided by Merck & Cie (Schaffhausen, Switzerland). All bulk ingredients have been compounded into vegetable gel-capsules at Natural Factors (Coquitlam, Canada). The prenatal vitamin contains the same micronutrient formulation as WN Pharmaceuticals Ltd® *Prenatal* (NPN 80025456), except the folic acid has been removed. Health Canada has reviewed and approved the use of the study supplements.

**Randomization and double blinding:** The randomization sequence was computer-generated using blocks of four which each contain two participants per supplement group. The list was provided to a research assistant who packaged the supplements into individual packs identified by participant study IDs. A research assistant at Natural Factors will store a document outlining codes assigned to (6S)-5-MTHF and folic acid. Neither the researchers or the participants will know what form of folate each code represents until the analysis phase of the trial. A master spreadsheet listing each participant's study ID and the folate code they have been assigned will be kept, and can be used for unblinding at any point if needed.

**Concomitant Medications**: Once enrolled, all other medications will be permitted during the intervention period, but must be reported to the researchers. The only exception will be folate/folic acid containing supplements/natural health products, which will not be permitted throughout the intervention period.

**Biochemical outcome measures:** A 3-hr fasting venous blood sample (~15 mL) will be collected at both baseline and endline visits. The blood analysis will include:

- *Serum and RBC folate* – Serum folate (nmol/L) reflects recent status or dietary intake; RBC folate (nmol/L) indicates longer term status (e.g. previous 3-4 months). We will assess serum and RBC total folate using microbiological assay, as globally recommended.[31-33]
- *Plasma unmetabolized folic acid* - Unmetabolized folic acid (nmol/L) is not incorporated into RBCs; rather, it accumulates and circulates in plasma.[21] Thus, it will be measured in plasma using LC-MS.[34]
- *Complete Blood Count:* Analysis will be performed using an automated hematology analyzer (Sysmex XNL550, Kobe, Japan).
- *Vitamin B12, vitamin B6, choline, betaine* – Total vitamin B12 (pmol/L) will be measured in plasma using an architect immunoanalyser. [35-37] Choline (µmol/L) and betaine (µmol/L) will

be measured in plasma using LC-MS. Pyridoxal-5'-phosphate (nmol/L), a marker of vitamin B6, will be measured using HPLC.[38]

- *Folate metabolites* – *S*-adenosyl-methionine, *S*-adenosyl-homocysteine, total homocysteine, methionine and cysteine will be measured in plasma using LC-MS[39-42]
- *Peripheral blood mononuclear layer cells (PBMC)* – DNA methylation differences will be measured in PBMCs and genomic DNA will be extracted from PBMCs for assessment of *MTHFR* (677 C>T, *rs*1801133, and 1298 A>C, *rs*1801131) and *DHFR* (*rs*1643649 and *rs*70991108) gene variants using PCR[44-46]

**Dietary assessment:** Total folic acid and folate intakes will be assessed using a validated food frequency questionnaire: Block Folic Acid/Dietary Folate Equivalents Screener (from NutritionQuest).[47] We will calculate total dietary folate equivalents (DFEs) and adjust final outcomes for any differences between groups.

**Anthropometrics:** Pre-pregnancy BMI will be calculated using self-reported pre-pregnancy weight and height. Participants will be weighed at baseline and endline visits for calculation of gestational weight gain throughout the intervention period, and total weight gain throughout pregnancy using the reported pre-pregnancy weight.

**Other data to be collected:**

*Via a structured questionnaire*: At basline we will collect: age, country of birth, ethnicity, education, occupation, household income/number of people supported by income, medical and medication history, reported pre-pregnancy weight and height, past pregnancies, smoking status, alcohol consumption, diet history, dietary supplement use. Participants will also be asked how they hear about the study, to inform the most effective recruitment strategies. At endline we will ask for general feedback regarding the trial process and confirm any changes in medical conditions or medication use since the baseline visit.

**Timeline:** The research will be conducted over a period of approximately 2.5 years, with recruitment beginning as soon as ethics approval is complete and an intervention period of 16-weeks.

**Data analysis:** Descriptive statistics will be used for study participant characteristics, prevalence of nutritional deficiencies, and dietary intake data. *Primary aim:* We will calculate the mean ± standard deviation for serum folate, RBC folate and plasma UMFA in each group at baseline and endline. The change from baseline to endline in each group will be evaluated using a paired t-test. As a secondary test, we will evaluate the difference in the mean change between groups using a two-sample-t-test. All tests will initially be completed on an intention-to-treat basis according to initial group allocation at baseline and without any imputation for missing data. Secondary per-protocol analyses will be conducted including those who fully completed the study and adhered to the study protocol. Exploratory analyses will be conducted using a multiple linear regression model, adjusting for explanatory variables (e.g. DFEs, exploratory biomarkers, demographic data). *Secondary aim:* Overall study participation rate will be estimated by dividing the total number of women who agree to

participate by the total number of women invited to participate. Weekly participation rate will be estimated by dividing the total number of women who agree to participate, by the total number of weeks it took to recruit them. Participant retention rate will be estimated by dividing number of women who complete the full trial by the total number of women enrolled. Most effective recruitment strategies will be determined by recording number of successful participant acquisitions per recruitment site and by asking women in the questionnaire how they heard about the study. Capsule counts will be used to calculate participants adherent to study protocol and the supplement diary will used to provide descriptive insight into barriers to adherence.

**Data privacy and storage:** A consent statement that fully clarifies the study's purpose, risks and procedures will be provided and explained to participants and will highlight the participants' right to withdraw at any point from the study. All participants will be given a unique study ID, which will be used on all study documents and data collection forms. All electronic documents will be encrypted and stored on a secure server at the BC Children's Hospital Research Institute, and all paper documents will be stored in a locked filing cabinet in Dr. Karakochuk's office at the University of British Columbia. Only the principal investigators (Karakochuk and Hutcheon) and the research coordinator (graduate student, Kelsey Cochrane) will have access to the data. Blood samples will be kept in a secure, locked freezer at the University of British Columiba, and stored for approximately 5 years after collection.

**Risk assessment:** Folic acid is provided in the dose currently recommended in Canada (0.4-1 mg/d); thus, is considered low risk.[8] Natural (6S)-5-MTHF is recognized as safe and is included in Canadian prenatal vitamins with no established tolerable upper limit (no risk of harm is currently known). Randomization will occur between 8-21 weeks gestation (after neural tube closure). Participants will be notified of any clinically deficient nutrition biomarkers in their results.

**Methods for reporting adverse and serious adverse events:** An adverse event (AE) is defined as any untoward occurrence in a participant administered an investigational product. The AE may be: i) a new illness, ii) worsening of a concomitant illness, iii) an effect of the study protocol, or iv) a combination of two or more of these factors. The use of the term 'AE' does not imply a relationship with the study product or with the clinical study. Adverse events fall into the categories of 'non-serious' and 'serious'. A serious adverse event (SAE) is one that: i) results in death, ii) is life threatening, iii) requires subject hospitalization or prolongation of existing hospitalization, or iv) results in persistent or significant disability or incapacity. Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a SAE when, based upon appropriate medical judgment, the event may require medical intervention to prevent one of the outcomes listed above. All other AEs are considered non-serious.

The period of observation for collection of AE/SAEs will begin upon enrolment of participants and will continue throughout the duration of the study. Participants will be instructed to report any suspected AE/SAEs to the research coordinator immediately. All AE/SAEs will be assessed by the qualified investigator for recommendations on next steps. Participants may be withdrawn and instructed to discontinue the study supplements immediately if deemed appropriate by the qualified investigator.

Documentation of an AE/SAEs requires that a separate report be completed by the principal investigator in each case. All AEs occurring during the study will be reported and recorded, regardless of seriousness or relationship to the study product. The following information will be documented in each case: i) participant study ID, ii) description of the event, iii) intensity/severity, iv) seriousness, and v) relationship to the study product. If a participant had to discontinue supplements due to an AE/SAE, the supplements will be collected immediately by the researcher and re-tested for quality control. If there is a safety issue identified with the supplements, all participants will be contacted and instructed to immediately discontinue supplement intake.

All AE/SAEs will be reported to the ethics committee and to Health Canada. Researchers will continue to follow participants who are withdrawn from the trial due to an AE/SAE until their symptoms have resolved or returned to baseline.

**Withdrawal criteria:** Criteria for participant withdrawal includes: miscarriage, use of folate/folic acid containing supplements, and following an adverse/serious adverse event if the event in has been deemed due to the supplements/trial intervention (as assessed by the qualified investigator). Participants may also self-withdraw at any time, may be lost to follow-up, or will be withdrawn if recommended by their physician. If participants choose to enter the study and then are withdrawn at a later time, all information about them collected up to that point, including data obtained from blood samples, will be retained for analysis in order to protect the integrity of the research, which may benefit future research participants and patients. However, no further information will be collected.

**Compensation for study participation**: To compensate participants for their time (approx. 1.5 hours), we will provide a detailed summary of their folate analysis and dietary intakes report, prenatal vitamins throughout the intervention period, and a $25 gift voucher presented at their baseline and endline visit (for a total of $50). Travel and parking costs associated with study visits will be covered (up to $20/visit).

**Significance:** This trial will provide the proof-of-principle that supplementation with (6S)-5-MTHF can effectively increase blood folate concentrations during pregnancy while resulting in lower levels of unmetabolized folic acid, as compared to synthetic folic acid. These pilot data are critical for the design of an adequately powered definitive trial of (6S)-5-MTHF supplementation, as an alternative to synthetic folic acid, during pregnancy. Ultimately, the definitive trial will inform the safest and most effective form of folate supplementation for Canadian women and their babies.

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## Appendix

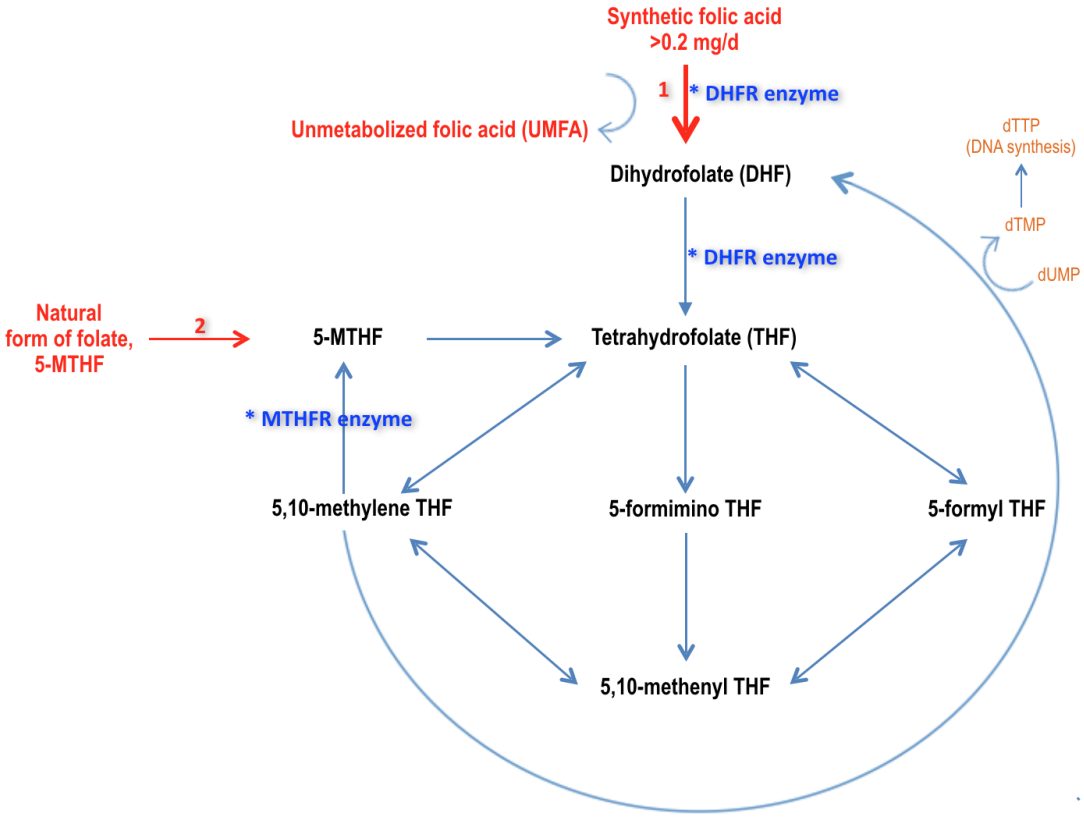


**Figure 1: Folate metabolism**

**1** - With doses of **synthetic folic acid >0.2 mg/d**, unmetabolized folic acid (UMFA) begins to accumulate in the blood. Excess synthetic folic acid and circulating UMFA can interfere with normal folate metabolism.
**2** - Supplementation with the **natural form of folate, 5-MTHF** does not require reduction or enzymatic activity (it is readily available for entry into the folate metabolism cycle) and does not interfere with folate metabolism.

## Table 1: Nutrition Facts for Study Supplements

| **Folate (participants will be randomized to either):** | **Dose: 1 capsule per day** |
|---|---|
| Group 1: Folic acid | 0.6 mg |
| Group 2: Natural folate | 0.625 mg |

*Non-medicinal ingredients: microcrystalline cellulose, magnesium stearate, purified water, hydroxypropyl methylcellulose, titanium dioxide

| **Prenatal Multivitamin Supplement (to be provided to all participants);** | **Dose: 2 capsules per day Per capsule (full dose)** |
|---|---|
| Beta-Carotene | 750 mcg (1500 mcg) |
| Vitamin A | 150mcg RAE (300mcg RAE) |
| Vitamin E | 6.75 mg (13.5 mg) |
| Vitamin D3 | 200 IU (400 IU) |
| Vitamin C | 42.5 mg (85 mg) |
| Niacin | 9 mg (18mg) |
| Pantothenic Acid | 3 mg (6mg) |
| Vitamin B6 | 0.95 mg (1.9 mg) |
| Vitamin B1 | 0.7 mg (1.4 mg) |
| Vitamin B2 | 0.7 mg (1.4 mg) |
| Biotin | 15 mcg (30 mcg) |
| Vitamin B12 | 1.3 mcg (2.6 mcg) |
| Calcium | 125 mg (250 mg) |
| Magnesium | 25 mg (50 mg) |
| Iron | 13.5 mg (27 mg) |
| Zinc | 3.75 mg (7.5 mg) |
| Manganese | 1 mg (2 mg) |
| Copper | 0.5 mg (1 mg) |
| Iodine | 110 mcg (220 mcg) |
| Molybdenum | 25 mcg (50 mcg) |
| Chromium | 15 mcg (30 mcg) |
| Selenium | 15 mcg (30 mcg) |

*Note the prenatal multivitamin is exactly the same as Prenatal (NPN 80025456), licence holder WN Pharmaceuticals Ltd®, except that it does not contain any folate.

**Non-medicinal ingredients: Microcrystalline cellulose, magnesium stearate, stearic acid, silicon dioxide, purified water, hydroxypropyl methylcellulose, titanium dioxide

**Table 2: Study Visits and Activities**

| Gestational age | Study visits (V) | Study activities |
|---|---|---|
| <21 weeks gestation | Recruitment: In-person, e-mail or phone | ▪ Confirmation of eligibility and enrolment<br>▪ Informed consent |
| 8-21 weeks gestation | **Visit 1**: Baseline<br>**Time**: About 60 mins | ▪ Baseline blood collection and questionnaire<br>▪ Food frequency questionnaire<br>▪ Randomization to folic acid or natural folate groups<br>▪ Distribution of supplements<br>▪ Weight/height |
| | | ▪ Intervention is ongoing (16 weeks total)<br>▪ Self-reported adherence diary |
| 24-37 weeks gestation | **V2**: Endline<br>**Time**: About 30 mins | ▪ Endline blood collection<br>▪ Weight<br>▪ Capsule count<br>▪ Endline Questionnaire |