CIRCULATING UNMETABOLIZED FOLIC ACID: RELATIONSHIP TO FOLATE STATUS AND EFFECT OF SUPPLEMENTATION

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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relationship to folate status and effect of supplementation

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ABSTRACT

There are increasing concerns that exposure to unmetabolized folic acid, which results from folic acid intakes that overwhelm the liver's metabolic capacity, may be associated with adverse effects. In this study, we examined the folic acid status of women of reproductive age in relation to dietary intake and the effect of folic acid supplementation (1.1 mg or 5 mg). Plasma unmetabolized folic acid was not significantly correlated with folate intake estimated by food frequency questionnaire or biomarkers. The proportion of women with detectable levels of unmetabolized folic acid increased from 65% to 100% after twelve weeks of supplementation (p < 0.05), however, the increase in concentrations did not reach statistical significance and the effect was not sustained. Moreover, there were no significant differences between the two doses. This suggests that there are mechanisms by which the body adapts to high folic acid intakes to limit exposure to unmetabolized folic acid.
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5,10-CH=H₂PteGlu  5,10-methenyltetrahydrofolate
5,10-CH₂-H₂PteGlu  5,10-methylenetetrahydrofolate
5-CH₃-H₂PteGlu    5-methyltetrahydrofolate
10-CHO-H₂PteGlu   10-formyltetrahydrofolate
AI                Adequate Intake
AICAR             aminomimidazole carboxamide ribonucleotide
AICART            aminomimidazole carboxamide ribonucleotide transformylase
AUC               area-under-the-curve
β-hCG             β human chorionic gonadotropin
CDC               Centers for Disease Control and Prevention
CI                confidence interval
DFE               dietary folate equivalent
DHFR              dihydrofolate reductase
DNA               deoxyribonucleic acid
DRI               Dietary Reference Intake
dTMP              deoxythymidine monophosphate
dUMP              deoxyuridine monophosphate
EAR               Estimated Average Requirement
EDTA              ethylenediaminetetraacetic acid
FAICAR            formyl-aminomimidazole carboxamide ribonucleotide
FBP               folate binding protein
FDA               United States Food and Drug Administration
FFQ               food frequency questionnaire
FGAR              formyl-glycinamide ribonucleotide
FIGlu             formiminoglutamate
FPGS              folylpolyglutamate synthase
GAR               glycinamide ribonucleotide
GART              glycinamide ribonucleotide transformylase
GC-MS             gas chromatography-mass spectrometry
GGH               γ-glutamyl hydrolase
H₂PteGlu          7,8-dihydrofolate
H₄PteGlu  5,6,7,8-tetrahydrofolate
Hct       hematocrit
HPLC      high performance liquid chromatography
LOAEL     lowest observed adverse effect level
LOD       limit of detection
MRC       Medical Research Council
MS        methionine synthase
MTHFC     methenyltetrahydrofolate cyclohydrolase
MTHFD     methylenetetrahydrofolate dehydrogenase
MTHFR     5,10-methylenetetrahydrofolate reductase
NHANES    National Health and Nutrition Examination Survey
NK        natural killer (cell)
NOAEL     no observed adverse effect level
NTD       neural tube defect
NVP       nausea and vomiting of pregnancy
PABA      para-aminobenzoic acid
PCFT      proton-coupled folate transporter
RBC       red blood cell
RDA       Recommended Dietary Allowance
RFC       reduced folate carrier
RP-HPLC   reversed-phase high performance liquid chromatography
RR        relative risk
SAH       S-adenosylhomocysteine
SAM       S-adenosylmethionine
SHMT      serine hydroxymethyltransferase
TS        thymidylate synthase
UL        Tolerable Upper Intake Level
WFR       weighed food record
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CHAPTER 1: INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Supplements and fortified foods contain folic acid, which is a fully-oxidized, non-coenzymatic form of folate. The process of converting folic acid to physiological forms of folate is initiated by dihydrofolate reductase (DHFR), which is expressed in the liver and other tissues. At low doses, ingested folic acid is completely converted to 5-methyltetrahydrofolate (5-CH₃-H₄PteGlu) in the liver. However, the liver has limited DHFR activity and doses above 260 to 280 µg saturate this conversion mechanism, resulting in the transient appearance of unmetabolized (oxidized) folic acid in plasma. There is increasing concern that high folic acid intakes may be associated with adverse health effects and that these effects may result from systemic exposure to unmetabolized folic acid. Since many women in Canada have high dietary folate intakes (as a result of fortification) and also consume folic acid supplements before and during pregnancy, a better understanding of unmetabolized folic acid in relation to other measures of folate status and the effect of folic acid supplementation is needed.

1.2 PURPOSE AND OBJECTIVES OF THE STUDY

The purpose of this study was to evaluate the folic acid status of healthy women of reproductive age in Canada and the effect of folic acid supplementation.

The specific study objectives were as follows:

(1) To examine the relationship between plasma concentration of unmetabolized folic acid and (a) dietary folic acid and total folate intake and (b) plasma and red blood cell total folate concentration.

(2) To examine the effect of folic acid supplementation on fasting plasma concentrations of unmetabolized folic acid.
1.3 RESEARCH HYPOTHESES AND RATIONALE

(1) Relationship between plasma concentration of unmetabolized folic acid and dietary folate intake and total folate concentrations in the blood.

Previous studies have shown dietary folate intake and blood total folate concentrations to be predictive of plasma folic acid. We, therefore, hypothesize that plasma folic acid will be higher among individuals who have (a) higher dietary folic acid and total folate intakes and (b) higher plasma and red blood cell total folate concentrations.

(2) Effect of folic acid supplementation on fasting plasma concentrations of unmetabolized folic acid.

The appearance of unmetabolized folic acid in plasma results from folic acid intakes that saturate the hepatic metabolic capacity. This has been shown to occur at doses above 260 μg. Other studies have suggested that unmetabolized folic acid concentrations increase in a dose-dependent manner and that there is an accumulative effect of repeated dosing. We, therefore, hypothesize that folic acid supplementation will increase plasma concentrations of unmetabolized folic acid and concentrations will be higher among women consuming 5 mg of folic acid daily compared to women consuming 1.1 mg of folic acid daily.
1.4 LITERATURE REVIEW

1.4.1 Folate

1.4.1.1 Structure and properties

The term “folate” describes the group of B vitamers that share the same vitamin activity based on the parent structure of folic acid. The parent structure consists of an aromatic pteridine ring joined by a methylene bridge to para-aminobenzoic acid (PABA), which in turn is attached to glutamic acid by a peptide bond (Figure 1) (1). Folate vitamers differ in the oxidation state of the pteridine ring and substitution on the N5 and/or N10 nitrogen atoms. In addition, a polyglutamate tail consisting of up to nine glutamate residues, each one joined via amide linkage to the γ-carboxyl group of the preceding residue, may be added (1,2).

By convention, the term “folic acid” refers specifically to the fully oxidized and most stable form of the vitamin that is used in supplements and fortified foods.

![Diagram of folic acid structure]

Figure 1: Structure of folic acid (1).
The three parts of the parent structure include: 2-amino-4-oxo-pteridine, p-aminobenzoic acid, and glutamic acid. Substitutions (one-carbon groups) occur at the N5 and/or N10 positions.
1.4.1.2 Sources and bioavailability

Humans cannot synthesize folate and must, therefore, obtain it from exogenous sources. Folate exists naturally in foods as reduced folate polyglutamate conjugates. Foods that are rich in naturally-occurring folates include liver, legumes, and leafy green vegetables. In addition, folic acid is added as a fortificant to certain foods. As of 2007, fifty-two countries worldwide had national regulations mandating folic acid fortification of wheat flour (3). Folic acid is also found in supplements and multivitamins.

In general, the bioavailability of folic acid is higher than that of the naturally-occurring food folates. Under fasting conditions, the bioavailability of folic acid approaches 100% (4). When consumed with food, folic acid (either supplemental or as a fortificant) is about 85% bioavailable (4). The bioavailability of naturally-occurring food folates depends on whether folate is present primarily as a monoglutamate or polyglutamate (with the former being more bioavailable) and the presence or absence of dietary and non-dietary factors that can facilitate or inhibit folate absorption; on average, it is estimated to be 50%, although it can be as high as 60 to 90% from some fruits and vegetables (5).

1.4.2 Folate pharmacokinetics

1.4.2.1 Absorption

Dietary folate polyglutamates are cleaved to their corresponding monoglutamate forms by a zinc-dependent brush border enzyme, glutamate carboxypeptidase II (2). The absorption of folic acid and reduced folate monoglutamates occurs throughout the length of the small intestine, although the majority of absorption occurs in the proximal segment (2). At least two mechanisms contribute to intestinal folate absorption. The first is a saturable, carrier-mediated, pH- and energy-dependent mechanism involving the proton-coupled folate transporter (PCFT; SLC46A1) (6,7). The second mechanism involves the reduced folate carrier (RFC), which functions as an anion exchanger (7). The PCFT and RFC differ in terms of their functional properties, specificity for oxidized and reduced folates,
and expression in the small intestine (Table 1). After a large (i.e., pharmacological) dose, passive diffusion likely contributes to folate absorption as well (4).

| Table 1: Properties of intestinal folate transporters (7). |
|------------------|----------------|
|                  | PCFT           | RFC            |
| Optimal pH       | Acidic         | Neutral        |
| Specificity      | Oxidized ≈ reduced | Oxidized < reduced |
| Expression in small intestine | Upper > lower | Ubiquitous |

The transfer of folates across the basolateral membrane of the intestinal epithelial cell into the submucosa is poorly understood. However, it is thought to occur through a combination of passive diffusion and carrier-mediated transport (8). From the submucosa, folates passively diffuse into the mesenteric veins and are transferred to the hepatic portal vein (9).

1.4.2.2 Hepatic metabolism and storage

The majority of folate in the portal circulation is taken up into the liver (4). Here, folic acid is reduced to 5,6,7,8-tetrahydrofolate (H₄PteGlu) and methylated to 5-methyltetrahydrofolate (5-CH₃-H₄PteGlu) (Figure 2), which is polyglutamylated for storage in the liver or released into the bile via the hepatic canaliculi or into the hepatic veins for delivery to the systemic circulation (8). Folates excreted into the bile may subsequently be reabsorbed from the small intestine. This process of enterohepatic circulation is thought to contribute to the maintenance of plasma folate levels, particularly during periods of dietary deprivation (2).
Figure 2: Absorption and metabolism of folic acid to 5-methyltetrahydrofolate.
Folic acid is taken up into the hepatocyte from the portal circulation. In the hepatocyte, folic acid is reduced to 7,8-dihydrofolate (H$_2$PteGlu), which is then reduced to 5,6,7,8-tetrahydrofolate (H$_4$PteGlu); both reactions are catalyzed by dihydrofolate reductase (DHFR). Serine hydroxymethyltransferase (SHMT) catalyzes the transfer of the β carbon from serine to the N5 nitrogen of H$_4$PteGlu, producing glycine and 5,10-methylenetetrahydrofolate (5,10-CH$_2$-H$_4$PteGlu). 5-methyltetrahydrofolate (5-CH$_3$-H$_4$PteGlu) is produced via reduction of 5,10-CH$_2$-H$_4$PteGlu by 5,10-methylenetetrahydrofolate reductase (MTHFR).
1.4.2.3 Distribution

Circulating folate exists almost exclusively as 5-CH$_3$-H$_4$PteGlu. Approximately two-thirds of plasma folate is protein-bound (4) and approximately 50% of bound folate is associated with low affinity binding proteins – mainly albumin. Other low affinity protein binders include α2 macroglobulin and transferrin (2). High-affinity folate binding proteins, including soluble forms of the folate receptors α and β, are also present in plasma (2). Under normal circumstances, plasma concentrations of these high-affinity binders are low; their expression is increased in various conditions, including folate deficiency and pregnancy (2).

1.4.2.4 Cellular folate uptake and efflux

Several mechanisms exist for folate uptake into cells and tissues, including carriers that are embedded in the cellular membrane and membrane-associated folate binding proteins (FBPs). The primary mode of transport into systemic tissues appears to be the RFC (8). However, the PCFT and membrane-associated forms of the folate receptors α and β appear to be involved in the re-absorption of folates from the renal proximal tubular lumen and in the transport of folates across the blood-brain barrier and placenta (8). The membrane-associated folate receptors are anchored to the cellular membrane via glycosylphosphatidylinositol-linkages and mediate folate uptake via endocytosis (8). Other membrane carriers that may be involved in cellular folate uptake and efflux include members of the organic anion transporter (SLC21) and organic anion/cation transporter (SLC22) families, multi-drug resistance protein (ABCC) family, and the breast cancer resistance protein (ABCG2) (8).

Folate enters the cell as 5-CH$_3$-H$_4$PteGlu. Inside the cell, methionine synthase (MS) transfers a methyl group from 5-CH$_3$-H$_4$PteGlu to homocysteine, forming H$_4$PteGlu and methionine. H$_4$PteGlu is the preferred substrate for folylpolyglutamate synthase (FPGS), which catalyzes the addition of glutamate residues to generate folate polyglutamates (2). This step is necessary for both the cellular
retention of folate and the conversion of folate to active coenzyme forms, as polyglutamates are better substrates for most folate-dependent enzymes compared to monoglutamates (2). The reverse reaction is catalyzed by γ-glutamyl hydrolase (GGH), which catalyzes the hydrolysis of the amide linkages between glutamate residues.

1.4.2.5 Elimination

The initial step in the breakdown of folate involves cleavage of the C9-N10 bond, producing para-aminobenzoylpolyglutamate and pterin (4,10). The pterin moiety is excreted in the bile, while the polyglutamate moiety undergoes further degradation (11). The polyglutamate is hydrolyzed to para-aminobenzoyleglutamate by a lysosomal GGH, which is then acetylated in the cytoplasm to produce acetamidobenzoyleglutamate (10). The acetylated derivative comprises the majority of folate catabolite excreted in the urine, although para-aminobenzoyleglutamate and unchanged folates may also be present, particularly at high intakes of folic acid (10).

1.4.3 Folate-dependent one-carbon metabolism

Folate participates as a coenzyme in one-carbon transfer reactions, which contribute to amino acid metabolism (glycine, serine, histidine), synthesis of purine (adenine, guanine) and pyrimidine (thymidylate) nucleotides, homocysteine-methionine metabolism, and the methylation cycle (Figure 3).
Figure 3: Folate-dependent one-carbon metabolism (11).
Folate enters the cells as 5-methyltetrahydrofolate (5-CH$_3$-H$_4$PteGlu). Methionine synthase (MS) and 5,10-methylenetetrahydrofolate reductase (MTHFR) are involved in homocysteine-methionine metabolism and the methylation cycle. Thymidylate synthase (TS) contributes to the synthesis of pyrimidine nucleotides. Methylene tetrahydrofolate dehydrogenase (MTHFD), methenyltetrahydrofolate cyclohydrolase (MTHFC), glycaminamide ribonucleotide transformylase (GART), and aminomimidazole carboxamide ribonucleotide transformylase (AICART) participate in the synthesis of purine nucleotide bases. Serine hydroxymethyltransferase (SHMT) and dihydrofolate reductase (DHFR) are involved in maintaining intracellular folate pools. SHMT is also involved in amino acid metabolism. 10-CHO-H$_4$PteGlu, 10-formyltetrahydrofolate; H$_2$PteGlu, 7,8-dihydrofolate; H$_4$PteGlu, 5,6,7,8-tetrahydrofolate; AICAR, aminomimidazole carboxamide ribonucleotide; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FAICAR, formyl-aminomimidazole carboxamide ribonucleotide; FGAR, formyl-glycinamide ribonucleotide; GAR, glycaminamide ribonucleotide.
1.4.3.1 *Amino acid metabolism*

The reversible interconversion of serine and glycine is catalyzed by serine hydroxymethyltransferase (SHMT), which requires pyridoxal-5′-phosphate (a derivative of vitamin B6) as a cofactor (11). SHMT catalyzes the transfer of the β-carbon of serine to H₄PteGlu, producing glycine and 5,10-CH₂-H₄PteGlu. The majority of one-carbon units that are used in folate-dependent one-carbon metabolism are derived from serine (11).

H₄PteGlu is also involved in the break down of formiminoglutamate (FIGlu), an intermediate in histidine catabolism (2). A bifunctional enzyme with glutamate formimidoyltransferase and formimidoyltetrahydrofolate cyclodeaminase activities catalyzes the transfer of a formimidoyl group from FIGlu to H₄PteGlu and subsequent removal of an amine group to produce glutamate and 5,10-methenyltetrahydrofolate (5,10-CH=H₄PteGlu).

1.4.3.2 *Nucleotide synthesis*

Folate is also important in the synthesis of purine and pyrimidine nucleotides for deoxyribonucleic acid (DNA) synthesis and repair. In purine synthesis, methyl groups derived from 10-CHO-H₄PteGlu become C8 and C2 of the purine ring (2). These reactions are catalyzed by glycinamide ribonucleotide transformylase (GART) and aminoimidazole carboxamide ribonucleotide transformylase (AICART), respectively. Synthesis of thymidylate (deoxythymidine monophosphate, dTMP) requires 5,10-CH₂-H₄PteGlu (2). Thymidylate synthase (TS) catalyzes the transfer of a single-carbon unit from 5,10-CH₂-H₄PteGlu to the 5′-position of deoxyuridine monophosphate (dUMP). Subsequent reduction of this carbon unit results in the formation of dTMP.

1.4.3.3 *Homocysteine-methionine metabolism and the methylation cycle*

In the homocysteine-methionine cycle, 5,10-CH₂-H₄PteGlu is reduced to 5-CH₃-H₄PteGlu by 5,10-methylenetetrahydrofolate reductase (MTHFR). Using 5-CH₃-H₄PteGlu as the methyl donor, MS catalyzes the conversion of homocysteine to methionine (11).
Homocysteine-methionine metabolism is also linked to the methylation cycle. Methionine is converted to S-adenosylmethionine (SAM), which is a cofactor for many methyltransferases that are involved in the methylation of DNA, proteins, and lipids (11). Methylation plays a critical role in regulating genomic stability and gene expression, neurotransmitter metabolism, and detoxification of drugs and chemicals (1). The loss of a methyl group from SAM produces S-adenosylhomocysteine (SAH). A low SAM/SAH ratio inhibits most SAM-dependent methyltransferases, thus SAH must be broken down for the methylation cycle to continue (1). SAH is hydrolyzed by adenosylhomocysteinase to adenosine and homocysteine.

1.4.4  Folic acid and pregnancy

1.4.4.1 Neural tube defects

Neural tube defects (NTDs) are congenital malformations produced by failure of the neural tube to form and close properly during embryonic development. The most common types of NTDs include spina bifida and anencephaly.

A relationship between folate deficiency and malformations of the central nervous system was first suggested in the 1960s by Hibbard and Smithells (12). A case-control study of NTDs and first trimester maternal vitamin concentrations found red blood cell (RBC) folate concentrations to be significantly lower among women who gave birth to an infant with a NTD compared to controls (13). Subsequently, two non-randomized trials of multivitamin supplementation among women who had previously delivered an infant with an NTD found the recurrence rate to be significantly lower among multivitamin-supplemented women compared to unsupplemented women (14,15). On the other hand, a small randomized trial reported by Laurence and colleagues yielded inconclusive results (16).

To clarify the issue, the Medical Research Council of the United Kingdom initiated a multicentre, double-blind, randomized controlled trial (the “MRC Vitamin Study”) to evaluate the effect of folic acid with or without other vitamins on the rate of NTD recurrence among women who
had a previous pregnancy complicated by a NTD (17). A total of 1817 women were randomized to one of four treatment groups: (a) folic acid, 4 mg; (b) multivitamin tablet containing vitamins A, B1, B2, B3, B6, C, and D; (c) tablet containing both folic acid, 4 mg, and other vitamins (i.e., those supplied in the multivitamin tablet); (d) tablet containing only the control substances, ferrous sulphate and dicalcium phosphate. This design allowed for the researchers to evaluate the effect of folic acid [(a) + (c) vs. (b) + (d)] and the effect of other vitamins [(b) + (c) vs. (a) + (d)]. Among 1195 informative pregnancies, the prevalence of NTDs among women allocated to receive folic acid was 1.0%, compared to 3.5% among women in the other groups. Thus folic acid reduced the risk of NTD recurrence by 72% (relative risk, RR, 0.28; 95% confidence interval [CI], 0.12–0.71). The effect of the “other vitamins” on the risk of NTD recurrence was non-significant (RR 0.80; 95% CI, 0.32–1.72).

In parallel to the MRC Vitamin Study, a single-centre, double-blind, randomized controlled trial (the “Hungarian randomized controlled trial”) was conducted to evaluate the effect of multivitamin supplementation on first occurrent NTDs (18). A total of 7540 women who were planning a pregnancy were enrolled and randomly allocated to one of two treatment groups: (a) multivitamin/mineral supplement containing 0.8 mg of folic acid; (b) trace element supplement. Among 4704 informative pregnancies, there were 6 NTDs – all in the trace element group (n = 2052). There were no NTDs among 2104 pregnancies in the multivitamin/mineral group (p = 0.029). The final report on this study included an additional 365 women (n = 7905) and an additional 749 informative pregnancies (n = 5453) (19) There were no additional NTDs in either group. The protective effect of the multivitamin (containing 0.8 mg of folic acid) on first occurrent NTDs was estimated to be 90% (20).

In 1999, Berry and colleagues reported on the effectiveness of folic acid alone (0.4 mg/day) in reducing the risk of NTDs (21). This evaluation was conducted as part of a public health campaign in one northern and two southern provinces in China. Comparing women who reported periconceptional use of folic acid to women who did not use folic acid at any time during the pregnancy, the relative risk for having a fetus with an NTD was 0.21 (95% CI, 0.10–0.43) in the southern regions and 0.59 (95%
CI, 0.36–0.97) in the northern region, providing further evidence that folic acid alone reduces the risk of first occurant NTDs.

Using data from a case-control study of NTDs in relation to maternal B-vitamin status (22), Daly and colleagues showed maternal folate concentration to be inversely associated with NTD risk in a continuous concentration-response relationship (Figure 4) (23). Based on their model, RBC folate concentrations above 906 nmol/L were highly protective, conferring a risk of 0.8 cases of NTDs per 1000 live births. Further, this represented an eight-fold reduction in the risk of having a fetus with an NTD compared to women with RBC folate concentrations of less than 340 nmol/L (150 ng/mL).

![Figure 4: Relationship between maternal RBC folate concentration and risk of NTD (23).](image)

1.4.4.2 Other congenital malformations

Periconceptional supplementation with multivitamins containing folic acid may also reduce the risk of congenital malformations. The strongest evidence exists for congenital heart defects (24-30) and, to a lesser extent, orofacial clefts (25,26,28,31). A small number of studies have also reported
a protective effect of multivitamin supplementation on limb defects (24,32-34) and urinary tract anomalies (25,32,34).

A systematic review and meta-analysis of 41 studies evaluating the effect of folic acid-containing multivitamins before and during the first trimester on rates of congenital malformations found supplementation to be associated with a reduced risk of NTDs, cardiovascular defects, and limb defects in both case-control studies and cohort and randomized controlled trials (35). For other congenital malformations, including orofacial clefts and urinary tract anomalies, a protective effect of multivitamin supplementation was observed in case-control studies, but not in cohort and randomized controlled trials.

1.4.4.3 Childhood cancers

Prenatal supplementation with folic acid or multivitamins containing folic acid has also been associated with a decreased risk of certain childhood cancers. Case-control studies of maternal diet and risk of pediatric brain tumours have found high folate intake and maternal multivitamin supplementation to be associated with a reduced risk of primitive neuroectodermal tumours, specifically neuroblastoma and medulloblastoma (36-38). These findings are further supported by a recent meta-analysis conducted by Goh and colleagues (39) and an interventional time series analysis that observed a decline in the incidence of neuroblastoma following the introduction of fortification (40). In contrast, Grupp and colleagues did not find a reduction in pediatric brain cancers after fortification in Ontario, although there was a 30% reduction in the incidence of Wilm’s tumour (41).

The association between maternal folic acid or multivitamin supplementation and risk of acute lymphoblastic leukemia, the most common type of childhood cancer, is less clear. Both positive (42,43) and neutral results have been reported in the literature (44-46). Two recent meta-analyses also produced conflicting results (39,45).
1.4.5 Measurement of blood folate concentrations

A number of methods have been developed to quantify folate in plasma, serum, and RBCs. Generally speaking, there are three types of folate assays: microbiological assays, immunoassays (folate binding assays), and chromatographic assays.

1.4.5.1 Microbiological assays

Microbiological assays are based on folate utilization by a test organism, usually *Lactobacillus rhamnosus*, using $^{14}$CO$_2$ production (47) or turbidity (48) as a marker of bacterial growth, which is proportional to folate content. Microbiological assays are considered by some to be the gold standard for folate measurement, due in part to their sensitivity and specificity for metabolically-active folates (49). However, the presence of certain medications, including antibiotics (e.g., penicillins, tetracyclines) and folate antagonists (e.g., trimethoprim, methotrexate), can inhibit bacterial growth, resulting in underestimation of the folate content (49). Microbiological assays also tend to be more time- and labour-intensive than other methods; therefore, while they are commonly used in research settings, they are less suitable for clinical applications.

1.4.5.2 Immunoassays

In a folate binding assay, the test sample is mixed with a known amount of labeled folate and high-affinity FBP; the binding of the labeled folate is quantified and compared to a standard curve to determine the folate concentration of the test sample (49). Binding assays are often automated or semi-automated, which contributes to their rapid turnover time and ease of use (50). Thus, in addition to their clinical applications, they are often used for population surveys, such as the National Health and Nutrition Examination Survey (NHANES) conducted by the CDC in the United States. It should be noted, however, that for the current NHANES, serum and RBC folate will be assayed using an automated microbiological method (D.L. O’Connor, personal communication).
The major limitation of folate-binding assays is marked variation in the affinity of FBPs for the various folate vitamers and (poly)glutamate chain lengths (51). Further, the presence of folate analogues and folate antagonists that bind FBPs, can lead to displacement of folate from FBP, resulting in overestimation of the folate content (49,51).

1.4.5.3 Chromatographic assays

Both microbiological methods and folate binding assays are highly sensitive and the former are highly specific to metabolically-active folates, however neither method is able to distinguish between folate vitamers. Therefore, chromatographic methods, which separate mixtures into their individual components, were developed to allow for quantification of individual folate vitamers. Separation of folate vitamers can be achieved by reversed-phase high performance liquid chromatography (RP-HPLC) (49,52). Folate vitamers are identified as they are eluted from the column, according to their characteristic retention times (52), and quantified by electrochemical detection, microbiological assay, or tandem mass spectrometry) (49). Given that such detail is not required for clinical applications, however, this method is essentially limited to the research setting.

Gas or liquid chromatography can also be used to measure total folate in samples by assaying PABA produced from the acid hydrolysis of folate (49). The quantity of PABA released from the test sample is measured by mass spectrometry or fluorescence detection and compared to the amount of labeled PABA released from a known concentration of a labeled folate standard. These methods are highly sensitive and the acidic conditions aid in the liberation of folate from plasma proteins and other specific and non-specific folate binders (49). On the other hand, gas chromatography-mass spectrometry (GC-MS), in particular, requires complex sample preparation that increases the likelihood of folate degradation (49). Other limitations of these methods include variation between folate vitamers in the efficiency of acid hydrolysis and the potential for interference by PABA derived from non-folate sources (e.g., some folate antagonists).
1.4.5.4 Agreement between analytical methods

In an international, round-robin interlaboratory comparison of microbiological assays, seven folate-binding assays, and an HPLC assay for serum and whole blood total folate, significant differences were observed between methods and between laboratories analyzing a common set of serum and whole blood samples (50). Variation between laboratories ranged from two- to nine-fold for serum samples and three- to nine-fold for whole blood samples, emphasizing the need for improved analytical accuracy and standardization. This led to the development of many of the newer chromatographic methods.

Clifford and colleagues evaluated the use of GC-MS for RBC folate, comparing it to a microbiological assay and two folate binding assays (radioimmunoassay, chemiluminescence) (53). RBC folate concentrations determined by radioimmunoassay and microbiological assay exhibited the greatest agreement with concentrations determined by GC-MS (r > 0.7, slope > 0.8). Studies comparing radioimmunoassay and microbiological assay to liquid chromatography-tandem mass spectrometry have generally found the microbiological assay to perform better than the radioimmunoassay (54-57). Taken together, these data suggest that the microbiological assay is the non-chromatographic method that provides the best estimates of RBC folate.

1.4.6 Recommended folate intakes

1.4.6.1 The Dietary Reference Intakes for folate

The Dietary Reference Intakes (DRIs) are a set of reference values of nutrient intakes for healthy populations. The DRIs generally comprise four values for each nutrient (Table 2) and consider not only the intake levels that prevent deficiency, but also intake levels that may reduce the risk of chronic disease.
**Table 2: Dietary Reference Intakes (4).**

<table>
<thead>
<tr>
<th>Reference value</th>
<th>Abbrev.</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated average requirement</td>
<td>EAR</td>
<td>Median usual intake that is estimated to meet the requirement of 50% of healthy individuals in a given life stage</td>
</tr>
<tr>
<td>Recommended dietary allowance</td>
<td>RDA</td>
<td>Average daily intake that is sufficient to meet the nutrient requirement of 97 to 98% of healthy individuals in a given life stage</td>
</tr>
<tr>
<td>Adequate intake</td>
<td>AI</td>
<td>Recommended average daily intake *</td>
</tr>
<tr>
<td>Tolerable upper intake level</td>
<td>UL</td>
<td>The highest level of daily intake that is likely to pose no risk of adverse health effects for almost all individuals in a given life stage</td>
</tr>
</tbody>
</table>

* Where there is insufficient evidence to determine an EAR and RDA, an AI is established. It considers observed or estimated intakes by a group (or groups) of healthy individuals.

For adults (19 to 50 years of age), a combination of RBC folate, plasma or serum folate, and plasma homocysteine were used to determine the EAR for folate (4). The primary focus was the folate intake needed to maintain normal concentrations of these biochemical indicators. Based on these data, an EAR of 320 μg/day was determined. An RDA of 400 μg/day was determined, assuming a 10% coefficient of variation in folate requirements.

Folate requirements increase dramatically during pregnancy, due to rapid cell division; thus a unique EAR and RDA were determined for pregnant women using RBC folate as a primary indicator of folate sufficiency. It was estimated that pregnant women required an additional 200 μg/day to maintain normal folate status, thus the EAR and RDA were set by adding this quantity to the values for non-pregnant women (520 μg/day and 600 μg/day, respectively) (4).

The DRIs for folate provide reference values as “dietary folate equivalents” (DFEs). The DFE was developed to provide a standard unit of folate that would account for differences in folate bioavailability between sources (Table 3).

**Table 3: Dietary folate equivalents (4).**

<table>
<thead>
<tr>
<th></th>
<th>Natural food folate</th>
<th>Folic acid from fortified foods</th>
<th>Folic acid from supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioavailability</td>
<td>50%</td>
<td>85%</td>
<td>100%</td>
</tr>
<tr>
<td>Amount that provides 1 μg DFE</td>
<td>1 μg</td>
<td>0.6 μg</td>
<td>0.5 μg</td>
</tr>
</tbody>
</table>
1.4.6.2 Recommendations for women who could become pregnant

On the basis of accumulating evidence supporting a reduction in NTD risk with periconceptional folic acid supplementation, including the MRC and Hungarian randomized controlled trials, the United States Public Health Service recommended that all women of childbearing age who were capable of becoming pregnant should consume 0.4 mg of folic acid daily. Further, women who were planning a pregnancy and who had a prior NTD-affected pregnancy were advised to consume 4 mg of folic acid daily beginning at least one month before conception and continuing through the first three months of pregnancy (58,59). These guidelines have persisted (with slight modification) and many agencies in other countries now issue similar recommendations (60).

Initially, there was no distinction between natural food folates, foods fortified with folic acid (optional at the time), and folic acid supplements – that is, women could choose to consume 0.4 mg of folic acid daily from food, supplements, or a combination of both. However, many agencies now recommend that women consume a daily supplement containing at least 0.4 mg of folic acid in addition to a well-balanced diet that includes fortified foods (61-63).

1.4.7 Fortification and the impact on folate status and rates of NTDs

1.4.7.1 Rationale for fortification

Efforts to increase dietary folate intake and use of folic acid supplements by women of reproductive age were met with limited success; in a Gallup Poll conducted for the March of Dimes in 1995, only 25% of respondents (women of reproductive age) reported taking a daily vitamin supplement containing folic acid (64). Therefore, in February 1996, the FDA announced the introduction of regulations that would require specific grain products, including flour and bread products labeled as “enriched,” to be fortified with folic acid (65). Compared to previous approaches, which required women to actively change their behaviour (i.e., by choosing more folate-rich foods and by obtaining and consuming folic acid supplements), fortification offered the advantage of providing
folic acid to a large number of individuals in the target population in a continuous and passive manner. Soon after, in December 1996, Canada moved to introduce a similar program (66,67). By the end of 1998, fortification of enriched flour and pasta was mandatory in both countries (Table 4).

<table>
<thead>
<tr>
<th>Table 4: Fortification levels in the United States and Canada (68).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>United States</strong></td>
</tr>
<tr>
<td>Wheat flour</td>
</tr>
<tr>
<td>Pasta</td>
</tr>
<tr>
<td>Cornmeal †</td>
</tr>
<tr>
<td>Rice †</td>
</tr>
<tr>
<td>Corn grits, farina †</td>
</tr>
<tr>
<td>Breakfast cereal †</td>
</tr>
</tbody>
</table>

† Optional; values indicated are permitted levels of fortification

The FDA considered three levels of fortification: 70 µg/100 g of cereal grain, 140 µg/100 g, and 350 µg/100 g (65). It was determined that a fortification level of 140 µg/100 g would provide the best balance between increasing folic acid intakes among women of childbearing age and keeping daily intakes among non-target populations below the UL of 1 mg/day. Several organizations, including medical associations, the Centers for Disease Control and Prevention (CDC), and the March of Dimes, argued for a higher level of fortification, citing models predicting that the mandated level of fortification would reduce NTDs by only 20%; however, when the implementation of fortification became stalled over the debate, the majority relented and petitioned the FDA to ratify the proposed level of fortification (69).

1.4.7.2 Blood folate concentrations

Data from the NHANES indicate that the fortification mandate has resulted in substantial improvements in blood folate concentrations in every segment of the United States population (70). The NHANES is a series of cross-sectional, nationally-representative examination surveys conducted by the National Center for Health Statistics of the CDC to assess health and nutritional status of adults and children in the United States. Among women of reproductive age (including pregnant women),
the median serum folate concentration increased from 10.9 nmol/L to 29.5 nmol/L between NHANES III (1988-1994) and NHANES 1999-2000; RBC folate increased from 363 nmol/L to 598 nmol/L (71). This was followed by a significant reduction in both serum and RBC folate concentration between NHANES 1999-2000 and NHANES 2003-2004, possibly due to an increase in the popularity of carbohydrate-restricted diets and a decrease in the amount of folic acid that was added to fortified foods (72). Data from NHANES 2005-2006 shows no significant change from the previous cycle (71). In the NHANES 2003-2004, the 90th percentile of RBC folate was 831.6 nmol/L, suggesting that achieving highly protective folate concentrations remains an issue for many women (72).

Fortification has also improved blood folate concentrations among Canadian adults and, specifically, women of reproductive age. Ray and colleagues reviewed all blood samples analyzed for RBC folate by a community medical laboratory in Ontario between April 1, 1997 and March 31, 2000 (73). Comparing the periods before (April 1 1997 to July 31, 1998) and after (February 1, 1999 to March 31, 2000) fortification, mean serum and RBC folate concentrations among adults increased from 18.5 nmol/L to 27.1 nmol/L and 680.3 nmol/L to 851.6 nmol/L, respectively. Among women of reproductive age, mean RBC folate concentrations increased from 527 nmol/L to 741 nmol/L (74). A similar increase in RBC folate was observed in another study of women in Newfoundland (75).

A recent report published by Bar-Oz and colleagues evaluated the folate status of women of reproductive age, using data collected from six clinical laboratories in the Greater Toronto Area (76). Between 1995 and 2006, mean RBC folate concentrations doubled, from 514 nmol/L to 1048 nmol/L and the proportion of women with RBC folate concentrations below 900 nmol/L decreased from 95.7% to 40.7% (Figure 5). The data were similar for a subset of women with a positive pregnancy test (i.e., elevated β human chorionic gonadotropin, β-hCG); the mean RBC folate concentration was 1113 nmol/L and 36% of women were below 900 nmol/L. These data suggest that, similar to the United States, there is room for improvement in improving the folate status among women of childbearing age in Canada.
Figure 5: RBC folate concentrations among non-anemic, normocytic women of reproductive age undergoing folate testing in Ontario (76).

1.4.7.3 Dietary folate intake

The fortification programs in Canada and the United States were expected to increase folate intakes by 100 µg/day among women of reproductive age. However, there is evidence to suggest that fortification may be providing up to two-times the intended amount. Using linear regression analysis to develop a regression equation relating folic acid intake to change in serum or plasma folate concentration, Quinlivan and Gregory estimated that, based on the change in blood folate concentrations, intake of folic acid from fortified foods exceeded 200 µg/day in the United States (77) and 150 µg/day in Canada (78).

Estimated dietary intakes based on traditional methods of dietary assessment have been more modest. Between NHANES III (1988-1994) and NHANES 1999-2000, mean dietary total folate intake (uncorrected for the higher bioavailability of folic acid) increased by an estimated 76 µg/day among adult non-supplement users, from 275 µg/day to 351 µg/day (79). Among women of
reproductive age, median dietary total folate intake increased by 74 to 156 μg/day, with the greatest increase observed among Hispanic women (80). Recent studies examining dietary folic acid intake among non-pregnant women in the United States have reported estimates ranging from 154 to 236 μg/day (81,82).

Estimates of dietary folic acid intake among women in Canada have been lower than those reported for women in United States. Liu and colleagues estimated by FFQ that mandatory fortification added an additional 70 μg/day of folic acid to the diet of women of reproductive age in Newfoundland (75). Using a food choice map (similar to an FFQ), Shuaibi and colleagues estimated dietary folic acid intake to be 96 μg/day among women 18 to 25 years of age in Winnipeg MB (83). Two other studies that used three-day WFRs to estimate dietary folate intakes among pregnant or nursing women in the Greater Toronto Area reported mean/median dietary folic acid intakes ranging from 118 to 147 μg/day (84,85).

1.4.7.4 NTD prevalence

A true estimate of the risk reduction relies on the complete ascertainment of all NTD cases, including those diagnosed in utero and after birth. In the United States, however, many birth defect surveillance systems do not document NTD cases diagnosed and terminated in utero, resulting in underestimation of the impact of their fortification program (86),(85). Estimates of the reduction in NTD prevalence have ranged from 19% (birth certificate data for live births only) (87) to 26% (combination of surveillance systems with and without prenatal ascertainment) (88,89). By comparison, with more complete case ascertainment, studies examining the effect of the fortification program in Canada have reported reductions in NTD prevalence of close to 50% (68,90).

Using antenatal maternal serum screening records, Ray and colleagues reported a 48% reduction in the prevalence of open NTDs (i.e., anencephaly and spina bifida) in Ontario following the introduction of mandatory fortification (90). Interventional time series analysis of the study period
observed a significant monthly decline in the combined prevalence of anencephaly and spina bifida. De Wals and colleagues assessed the yearly change in NTD prevalence in seven provinces in Canada between 1993 and 2002 (68). The study period was divided into pre-fortification (term births up to and including September 30, 1997), partial fortification (October 1, 1997 – March 31, 2000), and full fortification (term births on or after April 1, 2000). Infants included in the “full fortification” period were, therefore, conceived after the mother had been exposed to full fortification for a minimum of five months (by which time steady-state folate concentrations should have been achieved). Overall, the prevalence of NTDs (per 1000 births) decreased by 46%, from 1.58 in the pre-fortification period to 0.86 in the full fortification period. Provinces with higher baseline NTD prevalence (e.g., Newfoundland) observed greater reductions in rate, which was consistent with findings from the Chinese cohort study of prenatal folic acid supplementation (21).

1.4.8 Unique aspects of dietary and supplemental folic acid intake

1.4.8.1 Tolerable upper limit (UL)

The UL is defined as “the highest level of nutrient intake that is likely to pose no risk of adverse health effects for almost all individuals in the general population.” There is no UL for natural food folates, which are considered safe at any level of intake. Among healthy adults, the risk of toxicity from folic acid from fortified foods and supplements is also thought to be low, as it is a water-soluble vitamin and excess amounts are usually eliminated in the urine (91). At the same time, the Institute of Medicine Food and Nutrition Board has established a UL of 1 mg/day for folic acid from fortified foods and supplements based on the potential for high folic acid intakes to mask or exacerbate vitamin B12 deficiency (4).

The UL for folic acid was derived from a lowest observed adverse effect level (LOAEL), as there was insufficient data to determine the no observed adverse effect level (NOAEL). There were over one hundred reports where individuals with vitamin B12 deficiency received ≥ 5 mg/day of folic acid and
experienced a progression of the neurological symptoms, compared to only eight reports among
individuals receiving less than 5 mg/day of folic acid (4). Based on these data, 5 mg/day was
determined to be the LOAEL, which was divided by an uncertainty factor of 5 to arrive at the UL for
adults (including pregnant women) of 1 mg/day.

1.4.8.2 Unmetabolized folic acid

In contrast to natural food folates, folic acid is a non-coenzymatic form of folate. Folic acid
metabolism involves conversion of folic acid to coenzymatic tetrahydrofolate derivatives, primarily 5-
CH₃-H₄PteGlu. The first and rate-limiting step is catalyzed by DHFR, which reduces folic acid to
H₂PteGlu (and subsequently to H₄PteGlu) (92). The expression and activity of DHFR in human liver is
relatively low, such that oral doses greater than 260 to 280 µg can saturate the hepatic metabolic
capacity, resulting in the appearance of “unmetabolized” folic acid in plasma (93-95). With single
doses, however, folic acid is rapidly cleared from plasma through a combination of uptake into
peripheral tissues and renal excretion (93,96). On the other hand, two studies have demonstrated the
presence of detectable levels of unmetabolized folic acid in fasting plasma samples after eight to 14
weeks of supplementation with 400 µg/day of folic acid (97,98). This suggests that daily ingestion of
more than 400 µg of folic acid saturates not only hepatic DHFR activity, but also cellular uptake and
renal clearance mechanisms.

1.4.9 Public health significance of unmetabolized folic acid

There is much debate as to whether exposure to unmetabolized folic acid poses a health risk
(9,99,100). Theoretically, folic acid could interfere with normal folate metabolism through competition
with reduced, coenzymatic folates for transporters, binding proteins, and folate-dependent enzymes
(6,101-104). For instance, both folic acid and H₂PteGlu are substrates for DHFR. Although the affinity
of DHFR for H₂PteGlu is higher than its affinity for folic acid, in the presence of high concentrations of
folic acid, folic acid could competitively inhibit the conversion of H₂PteGlu to H₄PteGlu (105). As
neither folic acid nor H₃PteGlu is metabolically-active, this could theoretically create an intracellular folate deficiency (105). Another study observed a down-regulation of folate transporters in intestinal and renal epithelial cells cultured in growth media that was over-supplemented with folic acid (106). Although it remains to be seen whether these in vitro effects also occur in vivo, the potential implications of disturbed folate metabolism are wide-ranging; it is, therefore, critical that we gain a better understanding of the pharmacokinetics and pharmacodynamics of unmetabolized folic acid.

At present, there is no conclusive evidence that exposure to unmetabolized folic acid causes adverse health effects. However, potential concerns (i.e., those that appear to be uniquely associated with high folic acid intakes) include negative effects on vitamin B12 deficiency, cancer development, immune function, and epigenetic regulation.

1.4.9.1 Effects on individuals with vitamin B12 deficiency

In states of vitamin B12 deficiency, folate becomes “trapped” as 5-CH₃-PteGlu because of the impaired function of MS and because 5-CH₃-H₄PteGlu cannot be transformed back to its precursor, 5,10-CH₂-PteGlu. This is exacerbated by a reduction in SAM and loss of allosteric inhibition of MTHFR, resulting in even greater accumulation of 5-CH₃-H₄PteGlu (11,107). This “(methyl)folate trap” produces a pseudo-folate deficiency whereby folate is present in the cell but cannot participate in DNA synthesis or the methylation cycle. The inhibition of DNA synthesis leads to the hematological signs of vitamin B12 deficiency (i.e., macrocytosis and megaloblastic anemia); the neurological manifestations are thought to be due to inhibition of the methylation cycle and hypomethylation of myelin basic protein (107).

The mechanism by which folic acid is thought to mask vitamin B12 deficiency is by providing the cell with sufficient H₄PteGlu to bypass the “(methyl)folate trap,” thereby restoring DNA synthesis and normal hematopoiesis (108). However, while folic acid can correct the hematological abnormalities of vitamin B12 deficiency, it does not treat or prevent the demyelinating neuropathy
that results from prolonged or more severe deficiency. Although this was the primary concern raised during the period of scientific review that preceded the FDA’s decision to mandate fortification, most investigators now agree that there is a lack of quality data to suggest that “masked” vitamin B12 deficiency has increased since the introduction of fortification (9,100,109,110).

Some investigators now believe that exacerbation or progression of the hematological and/or neurological consequences of subclinical or clinical vitamin B12 deficiency may be the bigger concern (9,100). In a study of older Americans (≥ 60 years of age) using NHANES data collected between 1999 and 2002, a combination of high serum folate and low vitamin B12 was associated with a higher risk for anemia and cognitive impairment (111). By allowing the cell to bypass the “(methyl)folate trap,” folic acid restores the ability of the cell to synthesize DNA and to undergo cell division. However, the resultant increase in protein synthesis occurs at the expense of cellular methylation reactions (e.g., myelin synthesis), due to limiting amounts of methionine (108). Because the function of MS is already impaired in vitamin B12 deficiency, a further reduction in myelin synthesis could serve as the basis for exacerbation of neurological symptoms by folic acid supplementation. Alternatively, folic acid may act as a folate antagonist upon its conversion to H₂PteGlu (100). Accumulation of H₂PteGlu can inhibit TS and other folate-dependent enzymes involved in purine synthesis, resulting in impaired DNA synthesis. H₂-PteGlu also inhibits MTHFR; thus accumulation of H₂PteGlu could further impair homocysteine recycling and methionine synthesis.

The prevalence of vitamin B12 deficiency in women of reproductive age has traditionally been thought to be low (4). However, a recent report by Ray and colleagues suggested that approximately 1 in 13 women of reproductive age and 1 in 20 women in early pregnancy undergoing concomitant vitamin B12 and β-hCG testing in Ontario may be deficient in vitamin B12 (112). As low maternal vitamin B12 status is also a risk factor for NTDs (113,114), exacerbation of vitamin B12 deficiency could theoretically counteract the beneficial effect of folic acid supplementation on NTDs. However,
unless there is underlying malabsorption, this may be of minimal consequence, as prenatal multivitamins contain both folic acid and vitamin B12.

1.4.9.2 Folic acid and cancer

The folate requirement of all cells – and rapidly dividing cells, in particular – is the basis of antifolate therapy for cancer and immune conditions. It seems counterintuitive, therefore, that folate deficiency itself may also contribute to carcinogenesis. In vitro, animal, and human studies have shown low folate status to be associated with: (i) DNA hypomethylation, which could lead to activation of proto-oncogenes, and (ii) DNA strand breaks due to uracil misincorporation or defective DNA repair (115). These observations are supported by epidemiological evidence that tends to support a protective effect of folate on the development of cancer (115). At the same time, observations from animal studies suggest that folate may have a dual effect on cancer (99). That is, higher levels may inhibit the development of neoplastic lesions in normal tissues but facilitate the development and progression of established lesions.

Mason and colleagues examined the yearly incidence of colorectal cancer in relation to fortification in the United States and Canada (116). In both countries, the incidence of colorectal cancer had been declining for several years leading into the mid-1990s; however, around 1996 in the United States and 1997 in Canada, the trend reversed and the incidence of colorectal cancer began to rise, coinciding with the introduction of the legislation mandating fortification. The authors could not, however, rule out increased rates of screening and there were inconsistencies in the data (109).

On the other hand, two small studies of folic acid supplementation – including one that examined the use of 5 mg/day for three years – suggested that folic acid exerts a protective effect on recurrence of colorectal adenomas (117,118). The question of whether folic acid has a protective or detrimental effect on patients with a history of colorectal cancer has been the subject of three recent randomized, controlled trials (119-121). All three found no evidence of an effect of folic acid
supplementation overall. One study, however, suggested that folic acid supplementation could increase the risk of developing non-colorectal cancers and multiple (≥ 3) adenomas (119). On the other hand, one study suggested that folic acid supplementation could reduce the risk of recurrence among individuals with lower folate status at baseline (121).

Cancer outcomes have also been reported in four randomized, controlled trials of B-vitamin supplementation for the prevention of cardiovascular events (122-125). The dose of folic acid used in these studies was either 0.8 or 2.5 mg/day. The length of the follow up ranged from 40 months (median) to 7.3 years for the three completed trials; one trial was terminated prematurely (median length of follow up, 38 months). Two studies reported no significant difference in cancer risk when comparing patients who received the intervention (including 2.5 mg of folic acid) to patients who received placebo (122,125). In the other two studies, the incidence of cancer was higher among patients receiving 0.8 mg/day of folic acid (+ 0.4 mg/day of vitamin B12 +/- vitamin B6) compared to patients not receiving folic acid, but the difference was not statistically significant (123,124).

1.4.9.3 Effect on immune function

In a study examining the association between folate status and immune function among postmenopausal women in Seattle WA (126), Troen and colleagues found an inverse U-shaped relationship between folic acid intake from supplements and natural killer (NK) cell cytotoxicity, a marker of innate immune system function. Among women with lower dietary intake (< 233 µg/day), supplemental folic acid intake up to 400 µg/day was associated with increased NK cell cytotoxicity; with dosages above 400 µg/day, there was no significant difference compared to women who did not consume folic acid supplements. Among women with higher dietary intake (≥ 233 µg/day), folic acid supplements did not improve immune function; instead, NK cell cytotoxicity was significantly lower among women who consumed more than 400 µg/day of supplemental folic acid. There was no relationship between total
plasma folate concentration and NK cell cytotoxicity. There was, however, a significant inverse relationship between plasma unmetabolized folic acid and NK cell cytotoxicity.

As NK cells are a key component of the innate immune system and play a role in tumour cell destruction (127), the observed effect of folic acid on NK cell cytotoxicity may provide an additional mechanism by which folic acid could increase the risk for cancer.

1.4.9.4 Epigenetic effects

The developmental origins hypothesis postulates that the fetal environment influences an individual’s susceptibility to chronic disease later in life (128,129). Further, it is believed that epigenetic mechanisms, such as DNA methylation and histone modification, play a key role in the development origins of health and disease, as aberrant epigenetic regulation is certainly associated with cancer and some genetic disorders (e.g., Angelman syndrome and Prader-Willi syndrome) and may be associated with cardiovascular disease and metabolic disease (128,129).

In mammals, DNA methylation occurs on cytosine residues located 5’ to guanosine residues in CpG dinucleotides. Areas enriched in CpG dinucleotides (“CpG islands”) are often located proximal to the promoter regions of many genes. In general, hypermethylation of these CpG islands results in gene silencing; conversely, hypomethylation results in activation of gene expression. DNA methylation patterns are reprogrammed during embryogenesis, thus methyl donor nutrition could affect epigenetic regulation of the developing embryo (130). 5-CH$_3$-H$_4$PteGlu serves as a methyl donor in the conversion of homocysteine to methionine, which in turn serves as a methyl donor for many cellular methylation reactions upon activation to SAM. Animal studies have found periconceptional folate restriction or folic acid supplementation to result in changes in the fetal epigenome and the resultant phenotype of the offspring (131). Direct evidence of a similar effect in humans is lacking, although a recent study that found an association between maternal B-vitamin status and metabolic disease in the offspring offered an epigenetic dysregulation as a potential mechanism.
The Pune Maternal Nutrition Study is a cohort study that is being conducted in India to investigate the relationship between maternal nutrition during pregnancy and risk of type 2 diabetes and cardiovascular disease in children. It was recently reported that higher RBC folate concentrations and lower plasma vitamin B12 concentrations at 28 and 18 weeks of gestation, respectively, were significantly associated with increased insulin resistance, as determined using the homeostatic model assessment of insulin resistance (132). RBC folate concentrations were also positively associated with fat mass and percent body fat in the children at six years of age. The mechanism remains unknown, although the investigators suggested that nutritional reprogramming could occur as a result of altered DNA methylation and epigenetic regulation. Further research is needed to examine this possibility.
CHAPTER 2: MATERIALS AND METHODS

2.1 STUDY POPULATION

This blood samples and dietary data presented herein were collected as part of a prospective, randomized, open-label trial of 30 weeks of daily supplementation with 1.1 mg or 5 mg of folic acid as part of a prenatal/postpartum vitamin-mineral supplement (133). Participants were recruited through posters displayed at the Hospital for Sick Children (Toronto ON) and at designated locations on the St. George campus of the University of Toronto; advertisement in the Hospital for Sick Children newsletter; online advertisements published on the Hospital for Sick Children and Motherisk websites; and word-of-mouth. Motherisk is a clinical, research, and teaching program affiliated with the University of Toronto that provides evidence-based information on the safety of medications, infections, and other exposures during pregnancy and lactation.

All participants were healthy women between the ages of 18 and 45 at the time of study enrollment. Eligibility was assessed during an initial telephone or in-person interview. Potential participants were screened for the following conditions and excluded from participation, as appropriate:

• Use of folic acid supplements or multivitamin supplements containing folic acid in the six months preceding study enrollment
• Previous pregnancy in which an NTD was detected
• Family history of NTDs
• Concurrent use of medications known to affect folate status (e.g., antiseizure medications, folate antagonists, oral contraceptives)
• Allergy or hypersensitivity to any of the ingredients in PregVit® or PregVit-Folic5®
2.2 STUDY DESIGN

The protocol for the trial was approved by the Research Ethics Board at The Hospital for Sick Children (Appendix A).

2.2.1 Enrollment, randomization, and blinding

Eligible participants provided verbal informed consent to proceed with study enrollment; enrolled participants provided written informed consent at the first clinic appointment. Information collected during the enrollment process included (Appendix B):

- Contact information
- Demographic information (e.g., ethnicity, marital status, education, employment status)
- History of medication and substance use
- Medical and obstetrical histories

Randomization was performed by the Hospital for Sick Children research support pharmacists. Participants were randomized to receive either PregVit® (containing 1.1 mg of folic acid) or PregVit-Folic5® (containing 5 mg of folic acid). Neither the participants nor the study coordinator were blinded, as it was not feasible to modify the product appearance or packaging.

2.2.2 Study drugs

PregVit® and PregVit-Folic5® are vitamin-mineral supplements designed for use by planning and pregnant women. Both PregVit® and PregVit-Folic5® are formulated as two tablets that are to be taken daily: the pink (a.m.) tablet is taken in the morning and the blue (p.m.) tablet in the evening. The pink tablet and blue tablet contain different vitamins and minerals (Table 5); specifically, iron is supplied in the pink tablet and calcium is supplied in the blue tablet to facilitate iron absorption and reduce adverse events related to iron supplementation.
Table 5: Composition of study drugs, PregVit® and PregVit-Folic5®.

<table>
<thead>
<tr>
<th>Pink (morning) tablet</th>
<th>Blue (evening) tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (as beta-carotene)</td>
<td>2700 IU</td>
</tr>
<tr>
<td>Vitamin B1 (thiamin mononitrate)</td>
<td>3 mg</td>
</tr>
<tr>
<td>Vitamin B2 (riboflavin)</td>
<td>3.4 mg</td>
</tr>
<tr>
<td>Vitamin B3 (niacinamide)</td>
<td>20 mg</td>
</tr>
<tr>
<td>Vitamin B5 (pantothenate calcium)</td>
<td>5 mg</td>
</tr>
<tr>
<td>Vitamin B6 (as pyridoxine)</td>
<td>10 mg</td>
</tr>
<tr>
<td>Vitamin E (dl-α-tocopheryl acetate)</td>
<td>30 IU</td>
</tr>
<tr>
<td>Copper (cupric oxide)</td>
<td>2 mg</td>
</tr>
<tr>
<td>Iodine (potassium iodine)</td>
<td>0.15 mg</td>
</tr>
<tr>
<td>Iron (ferrous fumarate)</td>
<td>35 mg</td>
</tr>
<tr>
<td>Magnesium (magnesium oxide)</td>
<td>50 mg</td>
</tr>
<tr>
<td>Zinc (zinc oxide)</td>
<td>15 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>(PregVit®) 1.1 mg</td>
</tr>
<tr>
<td>(PregVit-Folic5®)</td>
<td>5 mg</td>
</tr>
<tr>
<td>Vitamin B12 (cyanocobalamin)</td>
<td>12 μg</td>
</tr>
<tr>
<td>Vitamin D3 (cholecalciferol)</td>
<td>250 IU</td>
</tr>
<tr>
<td>Calcium (calcium carbonate)</td>
<td>300 mg</td>
</tr>
</tbody>
</table>

2.2.3 Study procedures

Study visits were conducted in the Clinical Investigations Unit at the Hospital for Sick Children. All participants provided witnessed, written informed consent at the beginning of the first study visit (Appendix C).

At the first study visit, participants provided a fasting blood sample (5 mL) to measure baseline plasma and RBC folate and plasma vitamin B12. Participants were given an eight-week supply of their assigned multivitamins, which was renewed at subsequent study visits, and instructed to take the multivitamins as per the product monograph (i.e., pink tablet in the morning, blue tablet in the evening). Further instruction was given to leave missed or skipped doses in the blister packaging and to return all packaging, including unused tablets, to the study coordinator. Rates of adherence were determined based on the number of pills returned. Participants returned to the hospital at weeks 2, 4, 6, 12, and 30 (± 3 days) to provide fasting blood samples (5 mL) for plasma and RBC folate measurements. Plasma vitamin B12 was measured again at week 30.

A validated FFQ (53) was administered during the first and final study visits to assess usual dietary folate intake during the six months prior to study participation and during the 30 weeks (approximately seven months) of study participation, respectively.
2.3  BLOOD FOLATE ANALYSES

2.3.1  Blood sample preparation

Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-treated blood collection tubes (BD Vacutainer® K2 EDTA; BD Biosciences, Franklin Lakes NJ) after a minimum six-hour fast. Samples were shielded from light, placed near ice, and processed within two hours of collection.

To determine the hematocrit (Hct), whole blood was drawn into 75 mm heparinized capillary tubes (Allied Corp., Fisher Scientific; Pittsburgh PA) and centrifuged for 3 minutes (Hettich Haematokrit; Tuttingen, Germany). Hct was reported as the mean of at least two determinations.

Whole blood samples were prepared in triplicate in 2 mL polypropylene micro tubes (Sarstedt, Inc., Montréal QC). Aliquots (100 μL) of EDTA-anticoagulated whole blood were diluted 10-fold in 1% (wt:vol) ascorbic acid (A7631; Sigma-Aldrich Canada Ltd.; Oakville ON) in deionized water. Samples were vortexed and incubated at 37°C for 30 minutes to allow for lysis of red blood cells and deconjugation of polyglutamylated folates by plasma GGH.

The remaining whole blood was centrifuged at 1500 g for 20 minutes at 4°C (Allegra™ 21R Centrifuge; Beckman Coulter, Inc., Fullerton CA) to separate plasma from RBCs. The plasma layer was removed to a 14 mL polypropylene tube (Falcon™; BD Biosciences, Franklin Lakes NJ). Aliquots (500 μL) of plasma were transferred to two 2 mL polypropylene micro tubes for vitamin B12 analysis. Sodium ascorbate (134032; Sigma-Aldrich Canada Ltd.; Oakville ON) was added to the remaining plasma to a final concentration of 1% (wt:vol) to prevent oxidative degradation of folate.

All samples were frozen immediately after processing and stored at -80°C.
2.3.2 Affinity-HPLC assay for oxidized folic acid

Plasma concentrations of oxidized folic acid were measured by the affinity-HPLC method with electrochemical detection described by Bagley and Selhub (52,134) and Belz and Nau (135).

The affinity column consisted of immobilized FBP that was isolated from dried whey powder. To isolate FBP, 50 g of dried whey powder (ADM Neutraceuticals; Decatur IL) was suspended in 500 mL of water and the pH was adjusted to pH 9 with 5 mol/L sodium hydroxide. The suspension was refrigerated overnight and then centrifuged at 10 000 g for 30 minutes at 4°C. To prepare the column matrix, the supernatant fraction was allowed to react with Affi-Prep 10 affinity chromatography support (Bio-Rad Laboratories; Mississauga ON) overnight at 4°C. The FBP-Affi-Prep 10 slurry was washed sequentially with 20 mmol/L trifluoroacetic acid, 1 mol/L potassium phosphate, and deionized water. To prepare the column, 1 mL of the slurry was transferred to a glass Pasteur pipette packed with glass wool. Folate recovery from the prepared column, determined using $[^3]$H]-folic acid (Amersham Biosciences; GE Healthcare; Piscataway NJ), was 95 ± 2% (n = 10).

To prepare the samples for HPLC analysis, frozen plasma samples were placed in a water bath set at 100°C for 10 minutes to denature plasma proteins. Denatured samples were loaded on the prepared FBP-Affi-Prep 10 column, which was washed sequentially with deionized water to remove non-folate compounds and then mobile phase (equal proportions of A, B, and C; described in further detail below) to elute the purified folates.

Folic acid was quantified in affinity-purified samples by reversed-phase HPLC with electrochemical detection. The HPLC system consisted of a low pressure gradient pump (P580A LPG) fitted with an automated sample injector (ASI-100 Autosampler) set at 4°C to minimize sample degradation, phenyl analytical column (250 mm x 4.6 mm internal diameter, 5 μm particle size; BetaSil® Phenyl HPLC Column) installed in an oven set at 30°C (STH 585), and ED50 electrochemical detector with Ag/AgCl reference electrode managed by a computer running Chromeleon software.
(Version 6.2). The analytical column was purchased from Keystone Scientific (Thermo Fisher Scientific, Inc., Waltham MA). All other parts and software were purchased from Dionex Corp. (Oakville CA).

The mobile phase was delivered at a flow rate of 0.75 mL/min and maintained at 25% A (112 mmol/L potassium phosphate, 240 mmol/L phosphoric acid), 7% B (80% (vol:vol) acetonitrile in HPLC-grade water), and 68% C (HPLC-grade water) for the first 10 minutes. Between 10 and 40 minutes, the concentration of B was raised linearly to 20%, providing the gradient. The folic acid derivative was identified on the basis of retention time and comparison to the electrochemical response of the peak of the folic acid standard (F8798; Sigma-Aldrich Canada Ltd.; Oakville ON).

The limit of detection (signal-to-noise ratio = 3) of our HPLC set up was 100 pg of folic acid. Samples for which measured values were below the limit of quantification were spiked with a known quantity of folic acid standard and re-assayed.

Assay performance was evaluated using the standard curve that was generated at the beginning of each assay by injecting the folic acid standard in increasing volumes within the linear range of the assay. Injector precision and retention time reproducibility were within the specified limits (relative standard deviation ≤ 1%).

2.3.3 Microbiological assay for total folate

Plasma and whole blood total folate concentrations were measured using the microtitre plate method described by Molloy and Scott (48), with modification, using the test organism Lactobacillus rhamnosus (ATCC 7469; American Type Culture Collection, Manassas VA), which was reconstituted (thawed) daily from a cryopreserved stock.

To prepare the assay medium, 5.7 g of dehydrated assay medium (Difco™ Folic Acid Casei Medium; BD Biosciences, Franklin Lakes NJ) was reconstituted in 100 mL of deionized water. Ascorbic acid was added to a final concentration of 0.05% (wt:vol) and the mixture was heated. When the mixture was hot, but not boiling, 30 μL of Tween 80® (P8074; Sigma-Aldrich Canada Ltd.; Oakville ON)
was added and the mixture was brought to the boil for 2 to 3 minutes. After cooling slightly, 0.075 mg of ascorbic acid was added. The medium was shielded from light and held in an incubator at 37°C while the standards and test samples were prepared and deposited. When ready, thawed *L. rhamnosus* suspension (20 μL) was added to 50 mL of assay medium.

Frozen plasma and whole blood samples were thawed at room temperature, shielded from light. Thawed samples were diluted 80-fold in 1% (wt:vol) sodium ascorbate in deionized water. Aliquots of 20, 40, and 60 μL were deposited in triplicate. 1% sodium ascorbate was added to a volume of 100 μL followed by 200 μL of prepared assay medium.

Microtitre plates were covered with aluminum sealing tape (Corning®; Sigma-Aldrich Canada Ltd.; Oakville ON) and incubated at 37°C for 42 hours (Revco Ultima; Thermo Fisher Scientific, Inc., Waltham MA). After 42 hours, the plates were inverted and agitated to re-suspend the cells and the sealing tape was removed. The optical density at 590 nm was determined using a 96-well microtiter plate reader ( Opsys MR™; DYNEX Technologies; Chantilly VA) linked to a computer running the supplied software for data collection and analysis (Revelation Quicklink; DYNEX Technologies; Chantilly VA). Folate concentrations were determined based on the standard curve that was generated for each plate. The standard curve was based on a folic acid standard (0.5 ng/mL) that was prepared daily from a frozen stock and deposited in triplicate in aliquots of 0 to 100 μL (0 to 50 pg of folic acid).

All samples for a given subject were analyzed as a set to reduce intra-person variability. Measurements were discarded if the coefficient of variation for the triplicate exceeded 5% or if the measurements did not fall in linear range of the standard curve (7 – 21 pg); if no usable values remained for one or more weeks from a given subject, all samples from that subject were re-assayed, adjusting the dilutions as needed. Otherwise, reported values (in picograms) were plotted against the volume of the initial aliquot (i.e., 20, 40, and 60 μL); the slope of the line of best fit gave the folate concentration in the reaction well, which was multiplied by the dilution factor to determine the folate
concentration in the plasma or whole blood sample. RBC folate was calculated according to the following equation:

\[
RBC \text{ folate} = \frac{\text{whole blood folate} - [(1 - \text{Hct}) \times \text{plasma folate}]}{\text{Hct}}
\]

Assay performance (accuracy and inter-assay variability) were assessed using a certified whole blood folate standard (95/528; National Institute for Biological Standards and Control, Hertfordshire UK) that was analyzed in triplicate at two different dilutions on each plate. Reported values were checked against a quality control chart prepared in advance from twenty consecutive assays in which the standard was similarly analyzed. The acceptable limits were defined as ± 1 standard deviation from the mean of these twenty determinations. Our analyses yielded an overall inter-assay coefficient of variation of 3.4% and a measured concentration of 30.6 ± 1.0 nmol/L (stated value: 29.5 nmol/L; (136)).

2.4 DIETARY FOLATE ANALYSES

The Block Folic Acid/Dietary Folate Equivalents (DFE) Screener (NutritionQuest; Berkeley CA) was administered to assess dietary folate intake (Appendix D). The DFE Screener is an abbreviated folate-targeted food and supplement screening tool that was developed based on dietary data from NHANES 1999-2000 and designed to assess usual and customary folate intake in women (53). It includes 19 food groups and two supplement questions.

Questionnaires were processed and analyzed by NutritionQuest (Berkeley CA). The results were reported as:

(a) Naturally-occurring food folates (μg)

(b) Folic acid from folic acid-fortified foods (μg)

(c) Total food folate, μg (sum of (a) and (b))
(d) Total food folate, µg DFE (µg DFE = (a) + (b) x 1.7)

→ corrected for the higher bioavailability of synthetic folic acid

2.5 STATISTICS

2.5.1 Sample size and power

There was no sample size determination for the analyses presented herein as they were secondary to the main objective of the trial, which was to assess steady-state total folate concentrations achieved among women who supplemented daily with a multivitamin containing 1.1 mg or 5 mg of folic acid. As this was the first study to evaluate unmetabolized folic acid concentration among women of reproductive age, these data may be helpful in the planning of future studies.

With respect to the primary objective, the trial was designed to detect a difference in total folate concentrations that corresponded to a 40% difference in NTD risk reduction between the 1.1 mg and 5 mg doses with 80% power at an alpha level of 0.05 (133). The power of the secondary analysis of unmetabolized folic acid was not determined due to the lack of established methods for determining the power of non-parametric statistical tests, which were required by the non-normal distribution of unmetabolized folic acid concentrations.

2.5.2 Statistical analyses

Data were tested for normality using the Shapiro-Wilk test and parametric or non-parametric tests were performed as appropriate. All statistical analyses were performed using SAS for Windows (Version 9.1; SAS Institute, Inc.; Cary NC), except for the Friedman test due to the lack of post-hoc analysis options in SAS. Results were considered statistically significant at a p-value of ≤ 0.05.

Subject characteristics are presented as mean ± standard deviation or median (range). Between-group comparisons were performed using Student’s t-test, Wilcoxon-Mann-Whitney test, or Fisher’s exact test. Dietary folic acid intake and dietary total folate intake were compared between
groups and over time by using the MIXED procedure (PROC MIXED) in SAS for Windows. The MIXED procedure fits mixed linear models to data and estimates and tests the significance of between- and within-subject effects.

The relationships between plasma unmetabolized folic acid and (i) dietary folic acid intake, (ii) dietary total folate intake, (iii) total plasma folate concentration, and (iv) total RBC folate concentration were evaluated by calculating Kendall’s tau-b rank correlation coefficient, which measures association based on concordance and discordance between paired observations. Kendall’s tau-b was seen as preferable to Spearman’s rho due to the number of tied ranks (i.e., samples that were below the LOD).

Frequency data, including the proportion of women with detectable concentrations of unmetabolized folic acid, were analyzed by Fisher’s exact test for between-group comparisons or Cochran’s Q test for within-group comparisons (i.e., change over time). A significant Q statistic was investigated further by planned post-hoc pair-wise comparisons using McNemar’s test with Bonferroni correction for multiple testing to maintain a procedure-wise type I error rate of 0.05.

As unmetabolized folic acid concentrations were not normally distributed and it was not feasible to transform the data to fit a normal distribution, the effect of folic acid supplementation on plasma folic acid was analyzed using the Friedman test in WINKS SDA 6.0 (TexaSoft; Cedar Hill TX). A significant $\chi^2$ statistic was investigated further by non-parametric post-hoc pair-wise comparisons with Tukey adjustment for multiple testing.
CHAPTER 3: RESULTS

3.1 STUDY POPULATION

Between March 2007 and February 2008, sixty-three healthy, non-pregnant women of reproductive age were approached for participation in this study (Figure 6). Twenty-three women were excluded, either because they did not meet the inclusion criteria ($n = 21$) or because they did not wish to participate ($n = 2$); thus 40 women were enrolled. Twenty women were randomized to take the multivitamin containing 1.1 mg of folic acid; twenty women were randomized to take the multivitamin containing 5 mg of folic acid. One woman from each group withdrew from the study after the baseline measurement due to anxiety with the blood work ($n = 1$) or inability to commit to the study timeline ($n = 1$). Nineteen women in each group completed the study protocol and were included in the analyses.

![Figure 6: Consolidated Standards of Reporting Trials (CONSORT) patient flow diagram.](image-url)
There were no significant differences between the two groups of women in the collected patient characteristics (Table 6). Except for one woman who was a student pursuing post-secondary education, all of the women had earned a post-secondary degree (either college or university). The majority of the women were employed – either part-time or full-time. As per the inclusion criteria, all participants were healthy and were not taking any medications on a chronic basis. In the 1.1 mg folic acid group, one woman used acetaminophen or ibuprofen for infrequent migraine headaches and one used minocycline for acne on an “as needed” basis. In the 5 mg folic acid group, two women reported occasional use of salbutamol for asthma and one received desensitization shots for seasonal allergies. Occasional (social) alcohol consumption was reported by the majority of the women and did not differ significantly between the two groups. One woman in the 5 mg group reported light cigarette smoking.

<table>
<thead>
<tr>
<th>Table 6: Patient characteristics.</th>
<th>PregVit® (1.1 mg folic acid) (n = 19)</th>
<th>PregVit-Folic5® (5 mg folic acid) (n = 19)</th>
<th>p a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>33.4 ± 5.5</td>
<td>35.1 ± 7.0</td>
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</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td>54.5 (45.5 – 90.9)</td>
<td>61.8 (50.0 – 86.36) b</td>
<td>0.98</td>
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<tr>
<td><strong>Gravidity c</strong></td>
<td>0 (0 – 4)</td>
<td>1 (0 – 6)</td>
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<tr>
<td>Cigarettes</td>
<td>0</td>
<td>1</td>
<td>&gt; 0.99</td>
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</table>

a p-value, as determined by Student’s t-test, Wilcoxon-Mann-Whitney test, or Fisher’s exact test.
b Data was missing for one patient (i.e., n = 18).
c The proportion of women who had been pregnant before was not significantly different between the two groups (9/19 vs. 10/19; Fisher’s exact test, p > 0.99).
3.2 RELATIONSHIP BETWEEN PLASMA FOLIC ACID AND OTHER INDICATORS OF FOLATE STATUS

The relationships between plasma concentration of unmetabolized folic acid and dietary folate intakes and between plasma folic acid and total blood folate concentrations were evaluated by calculating Kendall’s tau-b rank correlation coefficient. Correlation coefficients were calculated within each group and for pooled data from both groups, as these analyses were performed on baseline data (i.e., samples collected before supplementation was started).

Plasma folic acid was not found to be significantly correlated with dietary folic acid (Figure 7) or dietary total folate (Figure 8) when analyzed by group or in pooled data. A significant negative correlation was observed between plasma folic acid and RBC total folate in the 1.1 mg group (Kendall’s \( \tau_b = -0.36, p = 0.04 \)), but not in the 5 mg group or in pooled data. After Bonferroni correction for multiple testing, however, the correlation did not retain statistical significance (critical p-value = 0.05 \( \div 12 = 0.004 \)). No other significant correlations were observed between plasma folic acid and plasma total folate (Figure 9) or RBC total folate (Figure 10).
Figure 7: Relationship between plasma folic acid and dietary folic acid intake.
1.1 mg group (triangles): Kendall’s $\tau_b = 0.12$, $p = 0.47$. 5 mg group (squares): Kendall’s $\tau_b = -0.18$, $p = 0.29$. Pooled: Kendall’s $\tau_b = -0.026$, $p = 0.83$.

Figure 8: Relationship between plasma folic acid and dietary total folate intake.
1.1 mg group (triangles): Kendall’s $\tau_b = 0.087$, $p = 0.61$. 5 mg group (squares): Kendall’s $\tau_b = -0.17$, $p = 0.32$). Pooled data: Kendall’s $\tau_b = 0.002$, $p = 0.99$. 
Figure 9: Relationship between plasma folic acid and plasma total folate.
1.1 mg group (triangles): Kendall’s $\tau_b = -0.26$, $p = 0.14$. 5 mg group (squares): Kendall’s $\tau_b = -0.073$, $p = 0.67$. Pooled data: Kendall’s $\tau_b = -0.16$, $p = 0.18$.

Figure 10: Relationship between plasma folic acid and RBC total folate.
1.1 mg group (triangles): Kendall’s $\tau_b = -0.36$, $p = 0.04$. 5 mg group (squares): Kendall’s $\tau_b = 0.15$, $p = 0.39$. Pooled data: Kendall’s $\tau_b = -0.068$, $p = 0.56$. 
Subjects were then grouped according to whether or not unmetabolized folic acid was detectable at baseline and compared on the same dietary and biochemical variables (Table 7). Neither dietary folic intake nor dietary total folate intake was significantly higher among individuals with detectable folic acid compared to those with undetectable levels; similarly, neither plasma nor RBC total folate concentrations were significantly different between the two groups.

Table 7: Baseline dietary and biochemical data for participants with detectable or undetectable (i.e., below the LOD) plasma concentrations of unmetabolized folic acid.

<table>
<thead>
<tr>
<th></th>
<th>Undetectable (n = 13)</th>
<th>Detectable (n = 25)</th>
<th>p (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma folic acid (nmol/L)</td>
<td>–</td>
<td>8.8 (0.27 – 41.9)</td>
<td>–</td>
</tr>
<tr>
<td>Dietary folic acid (µg/day)</td>
<td>188.5 (56.8 – 380.0)</td>
<td>191.8 (70.6 – 395.8)</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>Dietary total folate (µg DFE/day)</td>
<td>477.5 (181.9 – 849.2)</td>
<td>450.4 (231.9 – 979.4)</td>
<td>0.52</td>
</tr>
<tr>
<td>Plasma total folate (nmol/L)</td>
<td>48.7 (30.3 – 87.7)</td>
<td>43.7 (27.4 – 84.8)</td>
<td>0.27</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>969.2 (761.0 – 1777.5)</td>
<td>1018.61 (710.3 – 2355.1)</td>
<td>0.88</td>
</tr>
</tbody>
</table>

\(^{a}\) p-value, as determined by Wilcoxon-Mann-Whitney test (due to unequal sample sizes)
3.3 EFFECT OF FOLIC ACID SUPPLEMENTATION ON PLASMA CONCENTRATIONS OF UNMETABOLIZED FOLIC ACID

3.3.1 Adherence to multivitamin supplementation

Adverse events were reported by fourteen women (37%) over the course of 30 weeks of multivitamin supplementation, including nausea (n = 5), constipation (n = 3), abdominal discomfort (n = 3), diarrhea (n = 1), difficulty swallowing (n = 1), and heartburn (n = 1). All adverse events were mild in nature, however, and none of the women discontinued supplementation or withdrew from the study as a result of the event. Moreover, there was no significant difference in adverse events between the two groups.

The median rate of adherence was 88.8% (29.8 – 100%) in the 1.1 mg group and 89.8% (range 37.9 – 99.5%) in the 5 mg group (Figure 11). The difference was not significant (z = -0.37; p = 0.71).

![Box plot showing adherence rates](image)

Figure 11: Rates of adherence to multivitamin supplementation.
3.3.2 Proportion of plasma samples with detectable folic acid

The limit of detection (LOD) of our affinity chromatography-HPLC assay was 100 pg (0.18 nmol/L). Before supplementation, the proportion of women with a plasma concentration of unmetabolized folic acid that was above the LOD was 0.63 (95% CI, 0.39–0.83) in the 1.1 mg group and 0.68 (95% CI, 0.44–0.86) in the 5 mg group (Figure 12). There was a significant change in the proportion of women with detectable concentrations of folic acid over time in both the 1.1 mg group (Cochran’s Q = 33.89; df = 3; p < 0.001) and the 5 mg group (Cochran’s Q = 33.69; df = 3; p < 0.001). Post-hoc comparisons to baseline were statistically significant for week 6 and week 12 (p < 0.017; Table 8) but not week 30 in both groups (1.1 mg: p = 0.32, 5 mg: p = 0.32; Table 8). Comparing the proportions at each time point, there were no significant differences between the 1.1 mg and 5 mg groups (Fisher’s exact test; p > 0.99).

![Bar chart showing proportion of plasma samples with detectable folic acid over time](image)

**Figure 12: Proportion of plasma samples with detectable concentrations of unmetabolized folic acid.** Detection rates at week 6 and week 12 were significantly higher compared to baseline (week 0) in both the 1.1 mg (#) and 5 mg (§) groups.
Table 8: Within-group comparisons of the proportion of plasma samples with detectable concentrations of unmetabolized folic acid.

<table>
<thead>
<tr>
<th></th>
<th>McNemar’s S</th>
<th>df</th>
<th>p $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.1 mg group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0 vs. week 6</td>
<td>7.00</td>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td>Week 0 vs. week 12</td>
<td>7.00</td>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td>Week 0 vs. week 30</td>
<td>1.00</td>
<td>1</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>5 mg group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0 vs. week 6</td>
<td>6.00</td>
<td>1</td>
<td>0.014</td>
</tr>
<tr>
<td>Week 0 vs. week 12</td>
<td>6.00</td>
<td>1</td>
<td>0.014</td>
</tr>
<tr>
<td>Week 0 vs. week 30</td>
<td>1.00</td>
<td>1</td>
<td>0.32</td>
</tr>
</tbody>
</table>

$^a$ Critical p-value = 0.0167 after Bonferroni correction for 3 pair-wise comparisons within each group (0.05 ÷ 3 = 0.0167)

In the 1.1 mg group, of the seven women who had undetectable plasma concentrations of folic acid at baseline, all had undetectable concentrations of folic acid at week 30; of the 12 women who had detectable folic acid at baseline, eleven had detectable concentrations of folic acid at week 30 (kappa = 0.89; 95% CI, 0.68–1.00). A slightly lower level of agreement was observed in the 5 mg group – of the six women who had undetectable plasma concentrations of folic acid at baseline, five had undetectable concentrations at week 30; of the thirteen women who had detectable folic acid at baseline, ten had detectable concentrations at week 30 (kappa = 0.55; 95% CI, 0.18–0.93).

### 3.3.3 Plasma concentrations of unmetabolized folic acid

The distribution of plasma concentrations of unmetabolized folic acid was not normal, nor was it feasible to transform the data to fit a normal distribution due to the proportion of samples that were below the LOD at baseline and at week 30. The data were, therefore, analyzed using the Friedman test, a non-parametric equivalent to the repeated measures analysis of variance. The data were first analyzed by group (Figure 13).

At baseline, the median plasma concentration of unmetabolized folic acid was 4.8 nmol/L (undetectable to 41.9 nmol/L) in the 1.1 mg group compared to 3.7 nmol/L (undetectable to 22.7 nmol/L) in the 5 mg group ($z = 0.06; p = 0.95$). When analyzed by group, the change in plasma folic
acid over 30 weeks of supplementation was not significant in the 1.1 mg group ($\chi^2 = 4.71; \text{df} = 3; \ p = 0.20$) or the 5 mg group ($\chi^2 = 6.3; \text{df} = 3; \ p = 0.10$).

When pooled data from both groups were analyzed, the change in plasma folic acid was found to be significant (Figure 14; $\chi^2 = 10.39; \text{df} = 3; \ p = 0.019$). Post-hoc non-parametric multiple comparisons revealed a significant difference between plasma concentrations of unmetabolized folic acid at week 12 and week 30 ($Q = 4.04; \ p < 0.05$).

Comparing plasma folic acid between the two groups at each time point, there were no significant differences (Table 9).
Figure 13: Plasma concentrations of unmetabolized folic acid among women who supplemented with 1.1 mg of folic acid (grey boxes) compared to 5 mg of folic acid (cross-hatched boxes). When analyzed by group, the change in plasma folic acid concentrations was not significant in either the 1.1 mg (p = 0.20) or 5 mg (p = 0.10) group.
Figure 14: Plasma concentrations of unmetabolized folic acid among women who supplemented with either 1.1 mg or 5 mg of folic acid (all participants combined).
When pooled data from both groups were analyzed, there was a significant change in plasma folic acid concentrations (p = 0.019). (*) There was a significant decline from week 12 to week 30 (p < 0.05).
Table 9: Between-group comparisons of plasma concentrations of unmetabolized folic acid.

<table>
<thead>
<tr>
<th></th>
<th>Plasma folic acid (nmol/L)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.1 mg</td>
<td>5 mg</td>
<td>z</td>
</tr>
<tr>
<td>Week 0</td>
<td>4.76 (ND – 41.90)</td>
<td>3.67 (ND – 22.71)</td>
<td>0.06</td>
</tr>
<tr>
<td>Week 6</td>
<td>7.23 (1.79 – 81.92)</td>
<td>6.05 (0.18 – 28.98)</td>
<td>1.26</td>
</tr>
<tr>
<td>Week 12</td>
<td>8.34 (2.42 – 86.43)</td>
<td>8.02 (0.97 – 24.31)</td>
<td>-0.11</td>
</tr>
<tr>
<td>Week 30</td>
<td>3.13 (ND – 29.66)</td>
<td>4.35 (ND – 16.45)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

a p-value, as determined by Wilcoxon-Mann-Whitney test.

3.3.4 Estimated dietary folate intake

The Block DFE Screener was administered twice during the study – at the baseline study visit to estimate usual folate intake in the six months preceding study participation and at the final study visit to estimate usual folate intake over the course of the study. Differences between groups and over time were evaluated by mixed-model analysis of variance. There was no significant difference between the two groups at baseline or week 30 in dietary folic acid or dietary total folate intake (Tables 10 and 11); similarly, there was no significant change in dietary folic acid or dietary total folate intake from baseline to week 30 (Tables 10 and 11). Thus observed changes in unmetabolized folic acid were most likely due to the intervention, as there was no significant change in dietary intake.

Table 10: Estimated dietary folic acid intake (µg/day).

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 30</th>
<th>p (week 0 vs. week 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 mg</td>
<td>221.5 ± 93.9</td>
<td>218.9 ± 93.4</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>5 mg</td>
<td>194.3 ± 82.8</td>
<td>230.1 ± 116.3</td>
<td>0.22</td>
</tr>
<tr>
<td>p (1.1 mg vs. 5 mg)</td>
<td>0.78</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

* There was no significant effect of time (F(1,36) = 1.65; p = 0.21), group (F(1,36) = 0.08; p = 0.78), or time-group interaction (F(1,36) = 2.19; p = 0.15).

Table 11: Estimated dietary total folate intake (µg DFE/day).

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 30</th>
<th>p (week 0 vs. week 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 mg</td>
<td>525.6 ± 192.2</td>
<td>510.5 ± 191.5</td>
<td>0.97</td>
</tr>
<tr>
<td>5 mg</td>
<td>491.1 ± 185.6</td>
<td>541.2 ± 248.8</td>
<td>0.64</td>
</tr>
<tr>
<td>p (1.1 mg vs. 5 mg)</td>
<td>0.94</td>
<td>0.97</td>
<td></td>
</tr>
</tbody>
</table>

* There was no significant effect of time (F(1,36) = 0.43; p = 0.52), group (F(1,36) = 0.00; p = 0.98), or time-group interaction (F(1,36) = 1.47; p = 0.23).
CHAPTER 4: DISCUSSION, CONCLUSIONS, RECOMMENDATIONS

4.1 DISCUSSION

4.1.1 Characteristics of the study population

The women participating in this study were recruited primarily through advertisements posted in The Hospital for Sick Children and through word-of-mouth. Consistent with other studies conducted through the Motherisk program, the women participating in this study tended to be highly-educated and of higher socioeconomic status. All of the women were either enrolled in or had completed post-secondary education; the majority was employed full-time. As several studies have shown socioeconomic status, which encompasses education, employment, and income, to be a predictor of folate intake and adequacy (137-139), it was not surprising that most of the women (84%) had usual dietary folate intakes that met or exceeded the EAR for folate (320 μg/day DFE) from diet alone. Approximately three-quarters of the women met or exceeded the RDA of 400 μg/day DFE; none of the women exceeded the UL for folic acid. Estimated dietary total folate intakes (i.e., including natural food folates and folic acid-fortified foods) in our study group were similar to those reported previously among Canadian women of reproductive age (40,75,83-85,140). Dietary folate intakes remained relatively stable over the course of the study.

None of the participants were found to be folate deficient (serum/plasma folate < 7 nmol/L or RBC folate < 360 nmol/L). Consistent with a recent report on folate status of women of reproductive age in Ontario (76), about two-thirds of the women had RBC folate concentrations that are associated with a very low risk for NTDs (> 906 nmol/L); one-third of the women, therefore, would be at higher-than-baseline risk for NTDs, if they were to become pregnant.
4.1.2 Baseline concentrations of unmetabolized folic acid

To date, there have been two large population-based studies that examined circulating folic acid concentrations in a country with mandatory folic acid fortification. One study measured unmetabolized folic acid concentrations in plasma samples collected during the sixth examination cycle of the Framingham Offspring Cohort study, which took place between January 1995 and August 1998 (141); the second study measured folic acid in surplus serum samples collected from NHANES 2001-2002 participants ≥ 60 years of age (142).

The prevalence of detectable levels of unmetabolized folic acid in our population at baseline was similar to the prevalence in non-B vitamin users in the Framingham Offspring Cohort examined post-fortification (67%). In contrast, the median folic acid concentration in our study population was several times higher (3.76 nmol/L compared to 0.50 nmol/L). In the NHANES data set, both the detection rate (38%) and the mean folic acid concentration (1.7 nmol/L; the median was not reported) were lower compared to our population.

The underlying reasons for the apparent discrepancies between detection rates and plasma concentrations are unclear. We used an affinity-HPLC method with electrochemical detection based on the method described by Bagley and Selhub (52,134), as did the Framingham Offspring Cohort and NHANES studies. The reported detection limits were similar (0.18 nmol/L). However, as reviewed by Bailey and colleagues (142), available studies of unmetabolized folic acid (126,141-143) do not demonstrate a relationship between detection rates and LODs, suggesting that the observed discrepancies are more likely due to real differences between the study populations rather than methodological issues. Both the Framingham Offspring Cohort and NHANES studies examined an older population consisting of both men and women, whereas our population consisted of women of reproductive age. The NHANES population is heterogeneous and nationally-representative, whereas the Framingham Offspring Cohort and our population are more homogenous and self-selected.
Kalmbach and colleagues identified four predictors of “high” folic acid concentrations – dietary folic acid intake, dietary total folate intake, use of B-vitamin supplements, and plasma total folate (141). We excluded women who reported use of folic acid-containing supplements in the six months preceding study participation and the remaining three measures were not substantially higher in our study population compared to those reported for non-B vitamin users in the Framingham Offspring Cohort. Interestingly, these three measures also did not differ between participants with detectable and undetectable folic acid within our population, although the power of this comparison may have limited by the smaller number of women who had undetectable folic acid (n = 13). Another study described a possible “threshold” effect for serum total folate such that the proportion of serum folate as folic acid in samples above 50 nmol/L was found to be significantly higher compared to those below 50 nmol/L (54). Approximately one-third of the women in this study had a baseline plasma total folate concentration that was greater than 50 nmol/L; however, these thirteen women accounted for almost half of the plasma samples that did not have detectable levels of folic acid. Therefore the higher baseline folic acid concentrations in our population compared to the Framingham Offspring Cohort are not well explained by these factors.

Genetic factors likely contribute to inter-individual variation in folic acid metabolism. Of particular interest here is a common deletion polymorphism in intron-1 of the DHFR gene, which may contribute to the observed variability in DHFR activity (104). The molecular consequence of this 19 base-pair deletion is unclear, however, an in vitro study showed that proteins transcribed from DHFR genes lacking intron-1 are unstable and prone to lysosomal degradation (144). In the Framingham Offspring Cohort, the deletion polymorphism was associated with increased plasma concentrations of unmetabolized folic acid; specifically, among individuals with folic acid intakes above 500 μg/day, the prevalence of high concentrations of unmetabolized folic acid was twice as high among individuals homozygous for the deletion allele compared to heterozygotes or individuals homozygous for the wild-type allele (145). Differences in the distribution of DHFR genotypes and other genetic factors that
could influence folic acid metabolism might explain at least part of the variability between existing studies that have examined plasma folic acid concentrations.

Other possible explanations are differences in fasting requirements and noncompliance with fasting requirements. Previous studies have shown folic acid to be detectable in serum for at least four to six hours after dosing (93,98). However, assuming the half-life of folic acid in plasma is similar to the half-life of total folate, it would be necessary for subjects to fast for at least 10 hours to allow folic acid to be completely (97%) eliminated from plasma. Whereas subjects in Kalmbach and colleagues’ study were required to fast for a minimum of 10 hours, we only asked subjects to fast for a minimum of six hours. Most participants scheduled their appointments between 8 a.m. and 10 a.m. and completed the fast overnight, so it is likely that many women did fast for longer than the required six hours. However, participants were not specifically asked the timing of their last meal, therefore, we cannot say this with complete certainty. For the same reason, we cannot be certain that all women complied with the fasting requirement for all appointments. In a preliminary report on serum folic acid concentrations in the United States post-fortification, fasting length was the most significant determinant of circulating folic acid such that the concentration in non-fasting (< 3 hours) samples was on average 21.5 nmol/L higher than fasting samples (146).

4.1.3 Relationship between plasma folic acid and other measures of folate status

The majority of studies describing adverse effects of folic acid and/or folate have evaluated exposures in terms of high folic acid intakes or high plasma or serum folate concentrations. Circulating unmetabolized folic acid has only recently gained the attention of researchers, as prior to the development of chromatographic methods for measuring folate in biological samples, available methods (e.g., microbiological assays, folate-binding assays) did not distinguish between folic acid and reduced folates. With the advent of these chromatographic methods, studies have been able to show
that dietary folate intakes and blood folate concentrations are predictive of circulating folic acid. To date, however, these relationships have not been studied in a Canadian population.

4.1.3.1 Relationship between plasma folic acid and dietary folate intake

The majority of the women (79%) did not have usual dietary folic acid intakes that exceeded the threshold dose for the appearance of unmetabolized folic acid in plasma; none consumed more than 400 µg/day, which has been shown to produce a sustained appearance of folic acid in plasma (98). That folic acid was detectable in the majority of women in spite of low estimated folic acid intakes suggests that either (a) threshold doses are in fact lower than previously reported, or (b) estimated folic acid intakes provided by the Block DFE Screener were not accurate.

It is quite possible that threshold doses may be lower than the 260 to 280 µg (93) or 400 µg (98) reported previously – at least for some individuals. As briefly discussed in the previous section, the activity of DHFR, which is the rate-limiting step in the conversion of folic acid to 5-CH₃-H₄PteGlu, is highly variable. In a study using fresh human liver tissue, DHFR activity was found to vary approximately five-fold among samples (104). In two other studies by the same group, area-under-the-curve (AUC) and peak concentrations of unmetabolized folic acid were found to be highly variable following single doses of up to 5 mg of folic acid (147,148); in one study, peak concentrations varied over a ten-fold range among subjects consuming the same (0.4 mg) dose (148). Presumably, individuals with lower levels of DHFR activity would have lower thresholds and individuals with higher levels of DHFR activity could consume and metabolize larger doses of folic acid. Such variation in the metabolism of folic acid could explain why, contrary to our hypothesis, we did not observe a significant correlation between plasma folic acid and dietary folic acid or total folate intake.

In this study, dietary folic acid and dietary total folate intakes were estimated using the Block DFE Screener, a validated, folate-targeted, semi-quantitative FFQ designed to measure usual and customary intake of dietary and supplemental folate. It was designed as an instrument that would
rank subjects well according to folate intake and includes the 19 food groups that contributed to 60% of total folate intake in the United States in NHANES 1999-2000 (53). However, dietary patterns of Canadians and Americans may be quite different and the quality of food composition tables has been called into question (149-152), which could reduce the validity of this questionnaire in our population and attenuate the correlation with plasma folic acid.

A recent study of pregnant and post-partum women in Canada found that, similar to NHANES 1999-2000 data, grain products were the greatest contributors to dietary folate intake, followed by fruits and vegetables (79,84). These groups are well-represented in the Block DFE Screener, however, there are items included in the questionnaire that are not major contributors to Canadian intakes, including meal replacement drinks and bars, hot cereals, tortillas, and beer. Conversely, there are major contributors to dietary folate intake among Canadian women that are not represented in the Block DFE Screener, including dairy products and fast foods. The omission of these food groups could result in inaccurate estimates of folic acid intake for women who consume them.

Discrepancies between folate contents obtained from the United States Department of Agriculture Nutrient Database and actual amounts of folate in foods could also reduce the accuracy of estimated folate intakes. Both the Canadian and American fortification policies allow for overages – that is, the addition of extra vitamin or mineral to compensate for the loss or degradation of vitamins during shelf-life of the finished product – which can result in varying amounts of folic acid being present in the food at the time of consumption. In the first years after fortification was mandated in the United States, the amount of folate in foods was found to be, on average, double the regulation values (149). More recent data suggests that the folate content of breads has declined since then (150); however, a sizeable proportion of fortified foods may now have less folic acid than is mandated (151). In Canada, the amount of folate in foods has been found to be, on average, 50% higher than the values reported in the Canadian Nutrient File (152). Taken together, these data indicate that food
composition tables may be of variable quality and, consequently, dietary intake values calculated from these tables may be as well.

Limitations of FFQs in general must also be considered. In contrast to WFRs, which collect detailed quantitative and qualitative information about all foods and drinks consumed over a short period of time (153), FFQs sacrifice precise measurements of dietary intake on single days in exchange for more general information relating to intake over an extended period of time (154). As plasma concentrations reflect recent intake, the type of information captured by FFQs may be inadequate to assess the relationship between plasma folic acid and dietary intake. At the same time, Kalmbach and colleagues did find a significant linear relationship between plasma folic acid and dietary folic acid intake estimated by FFQ; however, as their FFQ included a more comprehensive food list, it likely provided more accurate and precise estimates of dietary intakes. Lastly, a potential correlation between plasma folic acid and dietary intake may have been attenuated by underreporting of dietary intake, which has been observed among overweight individuals and, to a lesser extent, women in general (155).

4.1.3.2 Relationship between plasma folic acid and blood total folate

As discussed above, the imprecision of the dietary assessment method used in this study may have contributed to the lack of correlation between plasma folic acid and dietary intake. The lack of correlation between plasma folic acid and plasma and RBC total folate, which are biomarkers of short- and long-term folate intake, respectively (156,157), is more difficult to explain.

The relationship between plasma concentration of unmetabolized folic acid and plasma total folate has been evaluated in several small studies and two large cohorts. Sweeney and colleagues measured plasma folic acid and plasma total folate in fasting plasma samples obtained from women undergoing elective Caesarean section and in non-fasting plasma samples obtained from a random sampling of individuals attending a blood donor clinic (143). In both populations, plasma folic acid and
plasma total folate were significantly correlated, although the strength of the correlation was stronger in fasting samples \((n = 20; r^2 = 0.300)\) compared to non-fasting samples \((n = 50; r^2 = 0.110)\). In contrast, Troen and colleagues did not find a significant correlation between the two measures in a study of the relationship between plasma folic acid and NK cell cytotoxicity among post-menopausal women in Seattle WA \((n = 105)\) (126). One cohort study evaluated the presence of detectable \((\geq 0.18 \text{ nmol/L})\) concentrations of unmetabolized folic acid in relation to serum folate (142); the second evaluated the presence of high (arbitrarily defined as \(\geq 1.35 \text{ nmol/L}, \text{ the 85}^{\text{th}} \text{ percentile for the cohort}\) concentrations of unmetabolized folic acid in relation to plasma folate (141). Both studies found a significant trend towards increasing odds of having detectable/high circulating folic acid with increasing quartiles of serum/plasma folate.

In general, studies that found an association between plasma folic acid and plasma or serum total folate included both supplement users and non-users. As a result, although dietary folic acid intakes were comparable to intakes in our population, mean or median total folic acid intakes (i.e., diet and supplements combined) were higher and the ranges of intakes were larger. The restricted range of dietary folic acid intakes in our population might explain the absence of correlation between plasma folic acid and plasma total folate. Because the median plasma total folate concentration in our study was comparable to, if not higher than, mean or median concentrations in previous studies, this further suggests that women in our study were achieving higher plasma folate concentrations as a result of higher intakes of naturally-occurring food folates – and not folic acid.

### 4.1.4 Effect of folic acid supplementation on circulating unmetabolized folic acid

Current guidelines advise all women who could become pregnant to consume a daily multivitamin containing 0.4 mg to 1 mg of folic acid (61-63). Women who are at higher risk for having a baby with a NTD are advised to consume a daily multivitamin containing 4 to 5 mg of folic acid, beginning at least three months before conception. Some authorities also recommend the high dose
strategy for women who have a history of poor medication adherence in addition to lifestyle issues that may increase their risk for NTDs (61).

Until recently, however, there was limited data on the pharmacokinetics of the higher dose of folic acid. Nguyen and colleagues were the first to formally investigate the single-dose and steady-state pharmacokinetics of the 5 mg dose of folic acid in women of reproductive age (133,158). As the high dose strategy provides more than ten-times the dosage that appears to saturate both hepatic metabolic capacity and plasma clearance mechanisms, we decided to expand on these findings by examining the effect of daily supplementation with 5 mg compared to 1.1 mg of folic acid on fasting plasma concentrations of unmetabolized folic acid. To the best of our knowledge, this was the first interventional study to evaluate the effects of long-term folic acid supplementation on plasma concentrations of unmetabolized folic acid among women of reproductive age who are also exposed to folic acid fortification.

4.1.3.2 Supplementation increases plasma concentrations of unmetabolized folic acid

We observed a significant increase in the proportion of women with detectable levels of unmetabolized folic acid over the first 12 weeks of supplementation; concentrations of unmetabolized folic acid also appeared to increase, however, although the overall effect of supplementation was significant, the differences between baseline and week 6 or week 12 were not statistically significant. This suggests that, although there is an effect of supplementation, it is small relative to the natural variation in circulating folic acid concentrations.

There is limited information on the effect of folic acid supplementation on plasma concentrations of unmetabolized folic acid. Bailey and colleagues have presented, in abstract form, preliminary data from a series of small trials evaluating the effects of 10 to 12 weeks of daily supplementation with 0.4 to 5 mg of folic acid in adults in the United States post-fortification (159-161). These studies included both men and women of varying ages and ethnicities. Plasma
concentrations of unmetabolized folic acid at baseline were, on average, 0.5 to 0.7 nmol/L. At the lowest dose tested (i.e., 0.4 mg/day), plasma folic acid increased approximately two-fold over 12 weeks of supplementation. With higher doses (i.e., 1 mg/day, 2.5 mg/day, or 5 mg/day), plasma folic acid concentrations increased approximately three-fold.

Similar to Bailey and colleagues’ findings, we also found that the median plasma concentration of unmetabolized folic acid doubled (approximately) over the first 12 weeks of supplementation; this was true in spite of baseline values in our population that were, on average, five-times higher. The discrepancy in baseline values is likely due in part to their exclusion of not only individuals who consumed folic acid supplements in the three months preceding their participation in the study, but also those who reported “significant” consumption of folic acid from dietary sources (e.g., fortified breakfast cereals, energy bars, etc.) (J.E. Ayling, personal communication). There are likely other differences between the study populations with respect to characteristics such as age, sex, and ethnicity – some of which may be associated with differences in folic acid metabolism.

It has been suggested that individuals who have detectable levels of unmetabolized folic acid may represent a subpopulation that has altered folic acid metabolism and responds differently to ingested folic acid (142). In our study, all of the women had detectable folic acid at some time over the course of supplementation; however, it is interesting to note that almost all of the women who had undetectable folic acid at baseline also had undetectable levels at week 30 and almost all of the women who had detectable folic acid at week 30 also had detectable levels at week 30. It is also interesting to note that, comparing the “detectable” and “undetectable” groups, there was no significant difference in dietary folic acid or total folate intake, suggesting that the women who had detectable folic acid at baseline and throughout the study may represent a “sensitive” group in our population. It would have been interesting to investigate the effects of folic acid supplementation among these women compared to the women who had detectable levels only during the interim study visits, however, our limited sample size precluded such analyses.
If such subpopulations do exist and were more highly represented in our study compared to Bailey and colleagues’ studies, this might also explain the differences in baseline concentrations of unmetabolized folic acid and response to supplementation.

4.1.3.3 Plasma concentrations of unmetabolized folic acid do not remain elevated

An unexpected observation was a significant decline in concentrations of unmetabolized folic acid between week 12 and week 30, despite ongoing supplementation and sustained total folate concentrations. In fact, plasma concentrations of unmetabolized folic acid at week 30 were not significantly different from concentrations at baseline. Preliminary data from a study that examined folic acid concentrations before and after six months of folic acid supplementation among women of reproductive age observed only minimal changes in folic acid concentrations with doses up to 4 mg/day (162), which is similar to what we observed in our population. Unfortunately, data were not available (or not collected) at smaller intervals over the course of supplementation, thus it is not known whether unmetabolized folic acid concentrations were significantly higher during the interim, as they were in ours (compared to week 30).

We first considered the possibility that adherence decreased over the latter half of the study. In a recent study of prenatal multivitamin supplementation in a cohort of Motherisk callers who had either discontinued a previous multivitamin or had yet to start multivitamin supplementation in pregnancy, the most common reasons for discontinuing or not starting supplementation were nausea and vomiting of pregnancy (NVP), difficulty with taking multivitamins, and adverse gastrointestinal events (163). Women in the present study, however, did not experience NVP (as they were not pregnant) and although approximately one-third of the women reported adverse events, all were mild in nature and did not result in discontinuation of the intervention or withdrawal from the study. In fact, the median rate of adherence approached 90% in each group. We did not obtain week-by-week or month-by-month records of pill intake, thus we cannot exclude the possibility that adherence was
higher in the initial weeks of the study before falling off towards the end; however, no decrease was observed in plasma or RBC total folate concentration (133). This suggests that pill intake occurred at relatively consistent rate over the course of the study and that the decrease in plasma folic acid was not the result of decreased adherence.

One possible mechanism for the observed decline in plasma concentrations of unmetabolized folic acid is up-regulation of folic acid metabolism (9,164). Kamen and colleagues found that, as compared to human liver cells in situ, the expression and activity of DHFR was 100- to 200-times higher in human cell lines in vitro and fresh rat liver cells. It was postulated that exposure to high levels of folic acid in culture medium and laboratory rodent chow up-regulated DHFR activity in cultured cells and in laboratory animals (165).

DHFR expression is partly controlled by a translational autoregulatory mechanism, where binding of DHFR to its cognate mRNA inhibits translation of the transcript (166). Binding of H₂PteGlu to the DHFR-mRNA complex induces a conformational change that releases the mRNA transcript, resulting in resumption of translation and DHFR synthesis. As the first step in the metabolism of folic acid to coenzymatic forms is the reduction of folic acid to H₂PteGlu, cells exposed to high levels of folic acid would likely accumulate high levels of H₂PteGlu as well. Thus folic acid, via reduction to H₂PteGlu, could theoretically up-regulate DHFR expression by translational de-repression. Further studies are needed to determine whether or not induction of DHFR by folic acid occurs in vivo and, if so, to what clinical outcome.

4.1.3.4 Effect of dose of folic acid supplementation

Contrary to our original hypothesis, plasma concentrations of unmetabolized folic acid were not significantly higher in the 5 mg group compared to the 1.1 mg group. This is consistent with preliminary data from the series of studies conducted by Bailey and colleagues that found no significant difference in folic acid concentrations achieved over 10 weeks of supplementation with
daily doses up to 5 mg (compared to 1 mg) (159-161). Although we cannot exclude the possibility that individuals consuming a supplement containing 5 mg of folic acid daily will temporarily be exposed to higher amounts of folic acid immediately after dosing, taken together, these data suggest saturation of folic acid uptake and/or retention and the existence of mechanisms that restore and maintain folate homeostasis following ingestion of pharmacological doses of folic acid. Nguyen and colleagues’ analysis of plasma and RBC total folate concentrations achieved in this trial of folic acid supplementation were also suggestive of a limiting mechanism, as only a two-fold difference in plasma and RBC total folate concentrations was observed despite a five-fold difference in dose (133).

At physiological doses, folic acid is absorbed via carrier-mediated transport involving the PCFT and RFC (7). Pharmacological doses, however, saturate carrier-mediated transport systems and are likely absorbed primarily via passive diffusion (4). As passive diffusion is a slower and less efficient means of absorption, this may explain the apparent non-linearity in steady-state pharmacokinetics of pharmacological doses of folic acid.

Another mechanism may involve down-regulation of the intestinal and/or renal transporters that are responsible for folate absorption and reabsorption, respectively. Using the Caco-2 cell line model of the intestinal epithelium and HK-2 cells (proximal renal tubule epithelial cells), Ashokkumar and colleagues observed that carrier-mediated uptake of tritiated folic acid by Caco-2 and HK-2 cells maintained in folate-oversupplemented media was significantly and specifically lower compared to cells maintained in folate-sufficient media (106). This was accompanied by significantly lower levels of RFC and PCFT protein in both intestinal and renal epithelial cells and of folate receptor protein in renal epithelial cells. This down-regulation appeared to be mediated in part via a transcriptional mechanism, as mRNA transcript levels and promoter activity were lower in folate-oversupplemented cells. The reduction in folate receptor protein is of particular interest to this discussion because it is unique among the three proteins studied in that it has a higher affinity for folic acid than it does for reduced folates (100). At least one-quarter of a 4 mg dose of folic acid is excreted unchanged as a result of
exceeding the renal capacity for reabsorption, which is mediated by the folate receptor (167). This would be exacerbated by a reduction in folate receptor expression and might offer a partial explanation for the absence of significantly higher levels of circulating folic acid in 5 mg group.

As the PCFT and folate receptor are also expressed in peripheral tissues, down-regulation of these transporters would theoretically lead to decreased cellular uptake of folic acid. Although this would lead to higher concentrations of unmetabolized folic acid in plasma, it would also predict a larger proportion of the dose being available for renal excretion. This may be compounded by saturation of cellular folate pools under conditions of high folate intake; as cellular folate concentrations increase, there is increased competition for FPGS and the marginal formation of polyglutamates decreases (168). Under these conditions, only a small proportion of folate that enters the cell is retained and the majority is released back into plasma.

In the primary analysis of this trial, Nguyen and colleagues found that women receiving the 5 mg dose of folic acid achieved significantly higher plasma and RBC total folate concentrations compared to women receiving the 1.1 mg dose (133). This, together with our finding that unmetabolized folic acid concentrations were not significantly higher, suggests that the higher total folate concentrations achieved with the 5 mg dose of folic acid constitute reduced, coenzymatic folates. Therefore, up-regulation of DHFR, as described in the previous section, may be another mechanism by which exposure to unmetabolized folic acid was regulated among women in the 5 mg group.

Finally, it is also possible that we did not observe higher unmetabolized folic acid concentrations in the 5 mg group because plasma samples were generally provided after a minimum six-hour fast. Assuming a half-life of approximately two hours (158), the majority (> 87.5%) of folic acid in plasma may have been cleared at the time of sampling. This, combined with the variability that we observed between individuals, may have rendered our study underpowered to detect a small difference in the amount of folic acid that might have remained at this time.
4.2 CONCLUSIONS

It is clear that folic acid supplementation is important for planning and pregnant women. What is not clear, however, is the optimal dose of folic acid needed for the prevention of NTDs and other folate-dependent congenital malformations. Current guidelines advise all women who “could become pregnant” to consume a daily multivitamin providing 0.4 mg to 1 mg of folic acid; a woman deemed to have personal characteristics or health conditions associated with an elevated risk of having a baby with a NTD may be advised to consume a higher dose of folic acid, depending on her contemporaneous folate status. However, there is a lack of research on the pharmacokinetics and safety of high dose folic acid supplementation.

Folic acid is generally considered to be safe at doses up to 1 mg/day and there is little evidence to show that doses up to 5 mg/day are harmful to healthy adults. In recent years, however, there has been increasing concern that exposure to unmetabolized folic acid, which results from folic acid intakes that overwhelm the body’s metabolic capacity, may be associated with adverse effects.

In Canada, legislation mandating fortification of enriched cereal grains with folic acid was introduced in 1998, resulting in universal increases in folic acid intakes and folate concentrations in the blood. In addition to consuming folic acid-fortified foods, many women also consume supplements containing folic acid, thus is important to develop a better understanding of the relationship between unmetabolized folic acid and dietary and biochemical indicators of folate status and the effect of supplementation.

In this study, we evaluated plasma unmetabolized folic acid in relation to dietary folate intake, blood total folate concentration, and the effect of supplementation among healthy women of reproductive age using plasma samples collected from a randomized trial comparing 30 weeks of supplementation with 1.1 mg or 5 mg of folic acid per day. To the best of our knowledge, this was the first clinical trial that was conducted for the purpose of evaluating the pharmacokinetics of high dose
folic acid supplementation in this population and the data presented herein are the first to describe
the folic acid status of women of reproductive age in a folic acid-fortified population.

In this study, we found that unmetabolized folic acid is present at low levels in the majority of
women who do not consume folic acid supplements but who do consume folic acid-fortified foods.
Contrary to our original hypothesis, however, there was no significant correlation between plasma
folic acid and dietary folic acid or total folate intake or between plasma folic acid and plasma or RBC
total folate in samples collected at baseline (i.e., before supplementation). The former may reflect
imprecision in our method of dietary assessment and the relatively restricted range of folic acid
intakes in our population. On the other hand, the latter suggests that the ability to metabolize folic
acid to reduced derivatives is highly variable and that this may be a more important determinant of
systemic exposure to unmetabolized folic acid.

Upon initiation of supplementation, we observed a significant increase in the proportion of
women who had detectable levels of unmetabolized folic acid in fasting plasma samples; however, the
increase was not sustained. A similar rise and fall was observed in the concentrations of
unmetabolized folic acid over the 30-week supplementation period, however, the increase in plasma
folic acid over the first 12 weeks of supplementation did not reach statistical significance. After 30
weeks of supplementation, both the proportion women with detectable folic acid and concentrations
of folic acid returned to levels that were not significantly different compared to baseline. For both
measures, there were no significant differences between the women receiving the 1.1 mg dose and
those receiving the 5 mg dose. These data suggest that there are homeostatic mechanisms that limit
systemic exposure to circulating folic acid, such as down-regulation of carrier-mediated transport
systems and up-regulation of folic acid metabolism.

Taken together with data previously published by our group, it appears that women who
supplement daily with 5 mg of folic acid achieve higher plasma and RBC total folate concentrations
compared to women who supplement with 1.1 mg/day without an apparent increase in exposure to
unmetabolized folic acid. This further suggests that the higher plasma and RBC total folate concentrations achieved with the 5 mg dose of folic acid represent reduced, coenzymatic folate, however, additional studies will be needed to confirm.

In summary, this thesis work both corroborates and contradicts current and common views on folic acid metabolism. For instance, hepatic DHFR activity in humans is considered to by highly variable but universally low. The variation in unmetabolized folic acid concentrations before and during supplementation that we observed supports the notion that hepatic metabolic capacity is highly variable; however, for many women in the present study, plasma concentrations of unmetabolized folic acid remained low even though they were consuming 2.5- to 12.5-times the daily dose that was previously shown to produce a sustained appearance of folic acid in plasma. For these women, it would appear that concerns surrounding excessive exposure to unmetabolized folic acid with the 5 mg dose may be unwarranted; thus further consideration could be given to the high dose folic acid strategy for the primary prevention of NTDs even in the absence of the standard risk factors. On the other hand, until more is known about the safety of exposure to unmetabolized folic acid, alternative approaches to achieving optimally protective folate concentrations could be considered for women who have a lower capacity to handle folic acid. One alternative could be supplementation with levomefolic acid (the calcium salt of 5-CH₃H₄PteGlu).
4.3 LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

This was the first study to examine plasma concentrations of unmetabolized folic acid in women of reproductive age in Canada, where we also have fortification of staple foods, and the effect of folic acid supplementation. Although we formulated research hypotheses, given the design of this investigation (i.e., as a secondary analysis) and the small sample size, this investigation was more suited to examine rather than to prove. Nevertheless, our findings and observations raise some important issues.

4.3.1 Development and validation of methods for measuring folic acid in plasma

We, and many others, use an affinity-HPLC assay for folic acid. However, there is currently no gold standard for measuring unmetabolized folic acid in plasma samples. Therefore, establishing a reference method and reference materials should be considered a priority for researchers in this area. As interest in unmetabolized folic acid grows, there will be a need to compare and contrast data from different groups using (potentially) different methods. Without an international standard, it will be difficult to determine if discordant study outcomes are due to differences in methodology or real differences between study populations.

4.3.2 Monitoring of folic acid concentrations

Our study population was self-selected and clearly not representative of the general population, thus the baseline prevalence of detectable folic acid and concentrations observed in this study cannot be extrapolated to women of reproductive age in general or to other demographic groups. However, based on the results of this study and others, it seems likely that the majority of individuals in countries with extensive fortification policies would be continuously exposed to low levels of circulating folic acid. Although the risks of exposure to unmetabolized folic acid remain theoretical at this point in time, the potential for serious adverse effects warrants continued
monitoring of folate status, including unmetabolized folic acid. Ideally, folate status would be monitored through national health surveys, such as the NHANES in the United States and the Canadian Health Measures Survey in Canada, so as to obtain nationally-representative data and to allow researchers to identify correlates and determinants of circulating folic acid and at-risk subpopulations.

4.3.3 Effect of folic acid supplementation in other populations

As mentioned above, this was the first study to quantify unmetabolized folic acid in Canada, however, the sample size was small and unmetabolized folic acid was not the primary outcome of the trial. Nevertheless, information from this study can be used in the planning of future studies. These studies should aim to examine larger and more representative samples, perhaps focusing first on the most vulnerable populations (e.g., older individuals) and the most likely to consume supplements containing folic acid (e.g., planning, pregnant, and nursing women). As a trial of folic acid supplementation for planning and pregnant women (analogous to the trial described herein) is ongoing, we may soon be able to identify and characterize differences – if any exist – between pregnant and non-pregnant women in the metabolism of folic acid.

It would also be of interest to examine the effect of folic acid supplementation in populations that are not also exposed to fortification. Individuals in Canada and the United States have been exposed to folic acid in fortified foods for over ten years; if such exposure has resulted in alterations in how we metabolize folic acid (e.g., up-regulation of DHFR), the effect of folic acid supplementation on the appearance of unmetabolized folic acid may have been attenuated. Studies in non-fortified regions will be needed to fully assess the impact of supplementation.
4.3.4 Statistical considerations

Plasma concentrations of unmetabolized folic acid were below the LOD in fasting plasma samples collected at baseline for approximately one-third of the women in our study. The relatively large number of “zero” values precluded transformation of the data to fit a normal distribution. Therefore, we were limited in the statistical analyses that we could perform. Specifically, we were limited to non-parametric statistical tests, which often have lower power compared to their parametric counterparts. Future studies will need to be much larger to overcome this limitation and to have sufficient power to detect within- and between-group differences.

4.3.5 Clinical significance of unmetabolized folic acid

In general, direct evidence of adverse effects of circulating folic acid is lacking. However, there are several potential harms for which there is biological plausibility and/or suggestive evidence from observational studies, including risk of cognitive impairment (with concurrent vitamin B12 deficiency), reduced innate immune function, and promotion of preneoplastic lesions (9). Future studies should aim to prospectively evaluate exposure to circulating folic acid in relation to these outcomes to determine whether or not unmetabolized folic acid is the causative agent, and if so, possible mechanisms and the nature of the dose/concentration-response.

4.3.6 Mechanisms of folate homeostasis

Our data suggest that there are mechanisms that limit exposure to unmetabolized folic acid; future studies should aim to characterize these mechanisms. This might be accomplished in a method similar to the one used by de Meer and colleagues, whereby the kinetics of a single dose of a labeled folic acid would be examined at regular intervals among individuals randomized to receive different doses of folic acid in a daily supplement (164). This could provide indirect evidence of physiological “adaptations” to chronic exposure to pharmacological doses of folic acid. For instance, if the plasma
AUC of labeled folate decreased over time, it would be indicative of decreased absorption or increased clearance; if the AUC of labeled folic acid decreased but the AUC of total folate remained the same over time, it would suggest increased metabolism of folic acid to reduced folate or specific clearance of folic acid. A study of this design would also be able to address the question of whether or not individuals consuming a higher dose of folic acid are temporarily exposed to higher levels of folic acid in plasma, which we were not able to ascertain in the present study.

The proposed study would need to be corroborated by direct evidence of the proposed adaptations (e.g., reduced expression and/or activity of folate transporters, increased expression and/or activity of DFHR). As it would be necessary to obtain tissue samples, such investigations would likely be most suited to in vitro, ex vivo, and animal models.
REFERENCES


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159. Bailey SW, Korzun WJ, Ayling JE. Persistent unmetabolised folic acid in plasma from subjects consuming 0.4 mg/day. Paper presented at: 3rd International Congress on Homocysteine Metabolism; July 1 – 5, 2001; Sorrento, Italy.


APPENDICES
Appendix A: Research ethics board approval for the randomized trial of folic acid supplementation
Dr. Melvin Freedman, REB Chair
555 University Avenue
Toronto, Ontario, M5G 1X8
Tel: 416-813-6152
Fax: 416-813-5085
Email: melvin.freedman@sickkids.ca

DATE OF APPROVAL: Nov 1 2006
EXPIRY DATE: November 2007
# Enrolment Intake Form

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## Patient Information

**Patient Name**

**Home Phone** ________________ **Work/Cell Phone** ________________

**Address**

**City** ________________ **Province** ________________ **Postal Code** ________________

**E-mail (optional)**

**Date of Birth** ________________ **Weight** ________________ kg/lbs

**LMP** ________________ **Is it regular?** □ Yes □ No

**Length of cycle** _____ days **Gravidity: G** ____ **P** ____ **SA** ____ **TA** ____ **Other**

**Part 3:**

**Planning a pregnancy?** □ Yes □ No

**Early in pregnancy?** □ Yes □ No

**GA** _____ **weeks** **EDC:** __________________
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<td></td>
<td>□ Liver disease</td>
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<td></td>
<td>□ Kidney disease</td>
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<tr>
<td></td>
<td>□ Other ___________</td>
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<tr>
<td><strong>Miscellaneous</strong></td>
<td>□ Hypertension</td>
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<tr>
<td></td>
<td>□ Diabetes</td>
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<td></td>
<td>□ Migraines</td>
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<tr>
<td></td>
<td>□ Anemia</td>
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<tr>
<td></td>
<td>□ Bacterial/viral infection</td>
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<tr>
<td></td>
<td>□ Asthma</td>
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<tr>
<td><strong>Psychiatric disorders</strong></td>
<td>□ Anxiety</td>
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<td></td>
<td>□ Depression</td>
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<td>□ Bipolar</td>
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<td></td>
<td>□ Other ___________</td>
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</table>
Appendix C: Consent form

Consent Form for Volunteers
Measuring Red Blood Cell and Serum Folate Concentrations Among Non-Pregnant Women of Childbearing Age: PregVit-Folic 5® vs. PregVit® Daily Supplementation

Name: ________________________________________________

Date of Birth: __________ / __________ / __________
( day ) ( month ) ( year )

Version date: March 29, 2007

Title of Research Project:
Optimizing Periconceptional and Prenatal Folic Acid Supplementation

Principle Investigator:
Gideon Koren, MD, Director of Motherisk Program, Hospital for Sick Children
Telephone: 416-813-5781

Purpose of Research:
We wish to measure red blood cell and serum folate concentrations among women of childbearing age, who do not practice multivitamin supplementation, before and after implementing daily multivitamin supplementation. We want to compare folate blood measurements between PregVit® and PregVit-Folic 5®. This may be important information for planning or pregnant women who need folic acid which has been shown to reduce the risk of neural tube defects and potentially other malformations.
**Description of Research:**

This is a two-arm comparison study: PregVit-Folic 5® (arm 1) contains 5 mg folic acid and PregVit® (arm 2) contains 1.1 mg folic acid. All other vitamin and mineral doses are identical between the 2 supplements. Both supplements are taken as 2 tablets daily, one tablet in the morning (am) and one tablet in the evening (pm). Both multivitamins are appropriate for periconceptional, prenatal, and post-partum supplementation.

The 2 arms are in equipoise which means they are considered equal and it is unknown which arm is better, hence the study is being conducted to determine if there is a difference.

This is a randomized study which means that a randomization process was used to assign, by chance alone, which group each participant belongs to. Thus, you were assigned by chance to the PregVit-Folic 5®/PregVit® group.

The following flow chart outlines the steps of participation:

- Research coordinator and potential participant discuss the study.
- Enrolment into study through Motherisk Program.
- Written consent and randomization.
- Participant comes to research site after a 6 hr fast.
- 5 mL blood sample will be drawn before multivitamin supplementation is initiated.
  (Measure baseline folate and vitamin B12 blood concentrations)
- Dietary folate questionnaire.
- Pick up first supply of multivitamin (with prescription) from Hospital for Sick Children pharmacy.
- Start taking the multivitamins everyday, at approximately the same time every day.
- Return to research site at weeks 2, 4, and 6 after starting the assigned multivitamin.
  Draw one blood sample each time.
- Pick up next supply of multivitamin at week 6 (hospital pharmacy).
- Return to research site at week 12 to draw one blood sample.
  Pick up next supply of multivitamin (hospital pharmacy).
- Return to research site at week 30 to draw last blood sample.
  Dietary folate questionnaire.
- Participation in the study is complete. Return all blister packs.
The total volume of blood that will be taken over the course of 30 weeks is approximately 40 mL (8 teaspoons).

Each appointment will be scheduled according to your menstrual cycle and when you are available.

To monitor self-administration of the multivitamins, we need you to return the blister packs and if possible, maintain a diary of pill intake (will be provided).

To document dietary folate, we will conduct telephone interviews regarding your diet for 7 days per month, over the course of your participation.

**Potential Harm and Discomfort:**

High doses of folic acid can mask vitamin B12 deficiency. However, this is generally not a concern for healthy individuals, with no chronic medical conditions. One study has shown that vitamin B12 deficiency can still be detected even with high folate blood concentrations. PregVit® and PregVit-Folic 5® both contain vitamin B12, thus it is being supplemented. Furthermore, vitamin B12 blood concentrations will be measured alongside folate blood concentrations to monitor for deficiencies.

The needle poking may not be pleasant. We will offer you a cream named EMLA® to massage on your arm, which takes away much (sometimes all) of the pain of poking. An alternative that can be used is a gel named Ametop®.

**Potential Benefits:**

We will be able to tell you your folate blood level. Results can be disclosed in person or mailed. Daily multivitamin supplementation can improve vitamin and mineral concentrations.

☐ I would like to know the results of the folate and vitamin B12 blood measurements.
☐ In person        ☐ By mail

**Alternatives:**

You are asked to volunteer for this study. There are no consequences if you do not participate.
Confidentiality

We will respect your privacy. No information about who you are will be given to anyone or be published without your permission, unless the law makes us do this.

Reimbursements:

We will reimburse you for your participation at $250 upon your completion of the study protocol. Under certain circumstances (as assessed by the study’s medical advisors), we will pro-rate payments depending on the degree of participation.

Participation:

Participation in research is voluntary. If you choose not to participate, you and your family will continue to have access to quality care at the Hospital for Sick Children. If you choose to participate in this study you can withdraw from the study at any time. Your participation may contribute to the creation of new diagnostic tests, new medicines or other events that may have commercial value. However, your participation in this study will not entitle you to a share in any future economic benefit.

Sponsorship:

The sponsor of this research is Duchesnay Ltd., Laval Quebec

Conflict of Interest:

Duchesnay Ltd. supports the Nausea and Vomiting of Pregnancy (NVP) Healthline at the Motherisk program, and Dr. Koren is a medical consultant for Duchesnay Ltd.
**Consent:**

“By signing this form, I agree that:
1) The study has been explained to me. All my questions were answered.
2) The possible harms and discomforts and the possible benefits (if any) of this study have been explained to me.
3) I know about the alternatives to taking part in this study. I understand that I have the right not to participate and the right to stop at any time. The decision about whether or not to participate will not affect my health care at the Hospital for Sick Children.
4) I am free now, and in the future, to ask any questions about the study.
5) I have been told that my medical records will be kept confidential, except where release of information is required by law, e.g., suspected child abuse, public health.
6) I understand that no information that would identify me, will be released or printed without asking me first.”

**I hereby consent to participate.**

The person who may be contacted about the research is:

Date ____________________________ Patricia Nguyen (research coordinator)

Name of Patient ________________________________ who may be reached at telephone #:

________________________

Age

**

Signature __________________________________________

416-813-7283 or by email:

patricia.nguyen@utoronto.ca

Name of person who obtained consent __________________________

Signature of person who obtained consent
### Improving Periconceptional and Prenatal Folic Acid Supplementation

**Block Dietary Folate Equivalents Screener**  
*First Write-Up before filling in the scantron*

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Study Participation:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Name</td>
<td>☐ Part 2. Measuring Serum and Red Blood Cell Folate Levels Among Non-Pregnant Women of Childbearing Age</td>
</tr>
<tr>
<td>Consultation Date:</td>
<td>☐ PregVit® Supplementation</td>
</tr>
<tr>
<td></td>
<td>☐ PregVit-Folic 5® Supplementation</td>
</tr>
<tr>
<td></td>
<td>☐ PregVit® Supplementation</td>
</tr>
<tr>
<td></td>
<td>☐ PregVit-Folic 5® Supplementation</td>
</tr>
<tr>
<td>Food</td>
<td>1x per month or less</td>
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<tr>
<td>-------------------------------------------</td>
<td>----------------------</td>
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<tr>
<td>Any cold breakfast cereal.</td>
<td></td>
</tr>
<tr>
<td>Any cooked cereals (i.e. oatmeal, cream of wheat, grits)</td>
<td></td>
</tr>
<tr>
<td>Eggs (including in breakfast sandwiches)</td>
<td></td>
</tr>
<tr>
<td>Rolls, bagels, muffins, hamburger buns</td>
<td></td>
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<tr>
<td>Bread slices (i.e. sandwiches, toast)</td>
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</tr>
<tr>
<td>Meal replacement drinks (i.e. Ensure, Carnation Instant Breakfasts)</td>
<td></td>
</tr>
<tr>
<td>Orange juice or oranges</td>
<td></td>
</tr>
<tr>
<td>Tea (brewed or iced tea) (not herb tea)</td>
<td></td>
</tr>
<tr>
<td>Crackers/cookies</td>
<td></td>
</tr>
<tr>
<td>Doughnuts, pastries, sweet rolls, cake, pan dulce, etc.</td>
<td></td>
</tr>
<tr>
<td>Tortillas (including burrito, enchilada, or other dish)</td>
<td></td>
</tr>
<tr>
<td>Beans (i.e. pinto, red/black beans, refried like in burrito)</td>
<td></td>
</tr>
<tr>
<td>Rice (or dishes with rice)</td>
<td></td>
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<tr>
<td>Spaghetti, pasta, macaroni, noodles</td>
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<tr>
<td>Pizza</td>
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<td>Green salad (i.e. lettuce, raw vegetables)</td>
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<tr>
<td>Spinach, chard, collards, mustard greens</td>
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<tr>
<td>Any other vegetables (i.e. string beans, peas, corn, broccoli, etc.)</td>
<td></td>
</tr>
<tr>
<td>Beer</td>
<td></td>
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</table>