Women and Reproduction: An Exploration of Factors Affecting Folate Status and Other Select Micronutrients Involved in One Carbon Metabolism

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
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Women and Reproduction: An Exploration of Factors Affecting Folate Status and Other Select Micronutrients Involved in One-Carbon Metabolism

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Doctor of Philosophy
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Abstract
Optimal function of one carbon (1-C) metabolism is necessary during reproduction as it supplies 1-C units for purposes of synthesis or methylation of nucleic or amino acids. Maintaining nutrient status of the methyl vitamins folate, choline, vitamins B₆ and B₁₂ is crucial to prevent the nutrient imbalances linked to increased risk of neural tube (NTD) and other birth defects. There is little understanding of whether or not pregnancy and folic acid (FA) supplementation influence 1-C metabolism—for example shunting toward DNA synthesis in pregnancy. Using red blood cell forms as a mechanism to evaluate partitioning of folate early in erythropoiesis we examined the RBC folate forms in pregnant women consuming 1 mg of FA and non-pregnant women consuming 0, 1 and 5 mg supplemental FA (n=26). We found that neither pregnancy nor level of FA altered the relative distributions of the folate in RBC indicating no preferment for DNA synthesis.

We then investigated if a comprehensive list of socio-demographic and lifestyle factors would improve our ability to predict if a woman was more likely to have RBC folate concentrations associated with NTD protection in women of childbearing years (WCBY), thereby removing the necessity for a blood sample (n=101). We found that a FA supplement of 200 µg/d or a multivitamin consumed
every other day, taking into account alcohol intake while controlling for ethnicity, can effectively
discriminate RBC folate concentrations associated with NTD protection 73% of the time.

Lastly, since the majority of women do not use multivitamins regularly, we systematically
investigated the specific foods that contribute to dietary folate, vitamin B_{12}, vitamin B_{6} and choline
intakes of WCBY using nationally representative data from the Canadian Community Health Survey 2.2
(n=4308). We found heavy reliance on FA fortified foods for folate intake and animal products (37-
94%) for B_{6}, choline and B_{12} intakes. The mean daily choline intake in WCBY was 238 mg with ~99 %
of WCBY having intakes below Adequate Intakes.

Our investigation suggests that status of the methyl nutrients is sensitive to a number of factors
influencing 1-C metabolism.
Dedication

I want to dedicate this thesis first to my parents Wilf and Jean Hartman, I am so grateful my father hung around long enough, even with all my delays, to be able to call me Doctor.

Secondly to my husband Harry Murray who put up with an abstracted wife during the writing process and remained my strongest supporter while I pursued my dream even when I doubted myself.

Lastly to my children Morgaine and Grant Craven who didn’t quite understand what I was doing while they were growing up but now are very proud of their mom.

I want to thank them for their faith, support and love. This was not the easiest journey as they well know but they helped me through it when I was questioning myself. I am grateful to have them in my life.
Acknowledgments

I wish to acknowledge my supervisor Dr Deborah O’Connor and thank her for her support, caring and the occasional kick in the pants during this rather convoluted time.

I wish to thank my committee members: Dr Pauline Darling, Dr Youngin Kim, Dr Michael Archer who graciously retired from my committee when my thesis went in a completely new direction, and Dr Anthony Hanley who joined us partway through and whose help has been invaluable.

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<th>Full Form</th>
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<tbody>
<tr>
<td>AI</td>
<td>Adequate intake</td>
</tr>
<tr>
<td>CCHS</td>
<td>Canadian Community Health Survey</td>
</tr>
<tr>
<td>CHMS</td>
<td>Canadian Health Measures Survey</td>
</tr>
<tr>
<td>CpG</td>
<td>CpG sites on DNA</td>
</tr>
<tr>
<td>DFE</td>
<td>Dietary folate equivalent</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated average requirement</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>MMA</td>
<td>Methylmalonic acid</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>NTD</td>
<td>Neural tube defect</td>
</tr>
<tr>
<td>PEMT</td>
<td>Phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PL</td>
<td>Pyridoxal</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal 5-phosphate</td>
</tr>
<tr>
<td>RDA</td>
<td>Reference daily intake</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
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<td>5-methyltetrahydrofolate</td>
</tr>
<tr>
<td>Compound</td>
<td>Fully Qualified Name</td>
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<tr>
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<tr>
<td>5-formylTHF</td>
<td>5-formyltetrahydrofolate</td>
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<tr>
<td>10-formylTHF</td>
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</tr>
<tr>
<td>5,10-methenylTHF</td>
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1. INTRODUCTION

1.1. INTRODUCTION

Folate is a term used to describe chemically related compounds which mediate the transfer of one carbon units for amino acid metabolism, the synthesis of nucleotides and the methylation of numerous bioactive compounds through intermediary metabolism (1). Methyl nutrients such as folate, vitamins B_6 and B_12 and choline are crucial for reproductive health due to their role in DNA/RNA synthesis and the production of S-adenosylmethionine (SAM), which is used in over 40 different methylation reactions in the body (2, 3). During reproduction, it is folate which is primarily associated with the reduction of neural tube defects (NTD), as well as the reduction of cleft lip and palate, megaloblastic anemia, and low birthweight (4-7). The etiology of NTDs is multifactorial and the protection mechanism associated with optimal folate intake, and to a lesser extent the intakes of vitamins B_6 and B_12 and choline, is still currently under debate. It has been shown conclusively that the addition of at least 800 µg folic acid (FA) as part of a multi-vitamin and mineral supplement can prevent the occurrence of NTD (8). Further, it seems there is a continuous dose-response relationship between red blood cell (RBC) folate concentrations and NTD risk with an 8-fold difference in risk among women with RBC folate concentrations < 340 nmol/L compared to ≥ 906 nmol/L (9). Public health campaigns promoting FA recommendations for women of childbearing years (WCBY) to consume supplemental FA prior to conception as a means of NTD prevention were implemented due to this evidence (10).

Adherence to these recommendations were initially very low, in part due to the fact that up to 50% of all pregnancies in Canada are unplanned (10-12). Therefore, it was determined that FA fortification of the food supply was the best approach to reaching the target population (WCBY) to maximize exposure (10, 12). Mandatory folic acid fortification in white wheat flour and grain products labelled enriched began in North America in 1998 (12, 13).

There is no doubt that FA fortification of white wheat flour products has been effective at reducing the prevalence of NTD in North America by 28-46% depending on the geographical area and accuracy of data collection (5, 6, 14). In conjunction with this decline, there has been a distinct shift upwards in serum and RBC folate concentrations by 2.5 and 1.5 times prefortification concentrations respectively, with less than 1% of the population having low serum (<10 nmol/L) or RBC concentrations (<340 nmol/L) (15, 16). Conversely, these same
studies show that up to 40% of the North American population have RBC concentrations above 1360 nmol/L,(15, 16), a cut-off suggested to be equivalent to the 97th percentile of the U.S. population prior to fortification (16, 17). Even with a significant number of individuals above the high cut-off, 22% of WCBY have RBC folate concentrations below a cut-off commonly associated with the reduction of NTD (≤ 906 nmol/L) (9, 16).

The percentage of individuals with high concentrations is of concern to some due to reports, albeit often inconsistent, that high intakes of supplemental FA and/or the associated increase in unmetabolized FA in serum (18, 19), may exacerbate effects of low B₁₂ status including cognitive impairment (20, 21), alter epigenetic fetal programming and cancer (22-24), and alter immune function (25, 26). Rodent models show the impact of high intake of methyl nutrients in maternal diets with epigenetic alterations in methylation patterns resulting in changes in coat mottling and colour, increased weight gain and affected glucose response (27-29). The impact of high folic acid intake in humans is more variable but reports, albeit inconsistent suggest an association with: altered DNA methylation and genetic selection (30-32), asthma and other respiratory disorders (33, 34), atopic dermatitis (35), increased insulin resistance and central adiposity (36, 37). The potential for epigenetic alterations in methylation patterns from high maternal FA intakes during gestation with the long term consequences not known, are of especial concern in WCBY due to the recommendations for additional FA supplementation in addition to the dietary intake of folate from both natural and fortified sources (38). While Health Canada recommends 400µg of supplemental FA daily, the Society of Obstetricians and Gynecologists of Canada had recommended up to 5 mg FA/day for those considered to be at increased risk of NTD or having supplementation compliance issues (39) although these recommendations are currently being updated. The majority of multivitamins contain 400 µg FA, adequate B₁₂ (20-25 mcg) and no choline, while most maternal vitamins contain 1 mg FA, adequate B₁₂ and no choline. Extended use of maternal FA containing supplements has the potential to increase maternal RBC folate concentrations. A recent examination of RBC folate concentrations during pregnancy using the 1999-2006 National Health and Nutrition Examination Survey (NHANES) data found that the median concentration was 1628 nmol/L with the 25th percentile at 1360 nmol/L suggesting that 75% of pregnant women in the U.S. have concentrations not only above the cut-off suggested for NTD protection but above the high cut-off of 1360 nmol/L (40). There are no recent national data of RBC folate concentrations of Canadian women during pregnancy,
but a small study (n=61) found median concentrations to be 3024 nmol/L at 36 weeks of gestation with the majority using ≥ 1 mg supplemental FA daily (41).

There is no doubt there has been a strong public policy focus in North America to ensure that women have adequate folate status during the reproductive years, and this has been accomplished primarily through folic acid fortification of the food supply and the use of FA-containing supplements (42). It is well-documented that there is an increased demand for folate during pregnancy due to the rapid fetal and uteroplacental organ growth; this can cause a 42% decline in maternal serum folate concentrations resulting in deficiency in the absence of a folic acid fortified food supply or use of supplemental FA (3). Further, it has been shown that women demonstrate increased plasma clearance, catabolism and excretion during pregnancy, and that low folate status enhances this rapid utilization of folate (2, 3, 43) yet the changes that occur to support this rapid rate of anabolic activity have not been fully explored.

At this point in time, we are unaware of any physiological shifts or metabolic adaptations that occur in one carbon (1-C) metabolism during pregnancy which would facilitate meeting the increased requirements or if any physiological shifts are associated with FA supplementation. One-C metabolism is governed by the intermediary folate metabolites with tetrahydrofolate (THF) as the central metabolite between the methylation and synthesis pathways (44). There is evidence of physiological shifts to 1-C metabolism either away or towards the synthesis pathways in various tissues, including RBCs (which is reflective of folate metabolism during erythropoiesis), secondary to dietary deficiencies (45, 46), environmental exposures (47) and abnormal anabolic conditions such as cancer (48, 49). These physiological adaptations are reflected in alterations in concentrations of intermediate folate metabolites such as 5-methylTHF (whose conversion to THF governs the methylation pathway), and 10-formylTHF and THF both of which provide substrate for purine and pyrimidine biosynthesis in 1-C metabolism. We theorize that if a physiological response did occur during pregnancy to accommodate increased DNA/RNA synthesis, we would anticipate a shift in 1-C metabolism from methylation to purine and pyrimidine biosynthesis which would result in a corresponding increase in the non-methyl folate forms. An understanding of how pregnancy and FA supplementation can influence the intracellular distribution of folate can provide information about any physiological adaptations that occur during reproduction or from pharmacological doses of FA.
The most direct method to ascertain if women have adequate folate status to reduce the risk for folate-mediated NTDs and avoid prescribing unnecessarily high doses of FA would be to directly measure RBC folate concentrations. However, the feasibility of applying this method on a population level for WCBY is unrealistic given the expense of such an approach (50), and the lack of a definitive folate concentrations cut-off for reduction of NTD; therefore, tools to help predict if a woman is likely to have a folate status associated with NTD protection are required. There is little doubt that consuming a FA-containing supplement can significantly increase RBC folate concentrations to improve folate status to reduce risk of NTDs (42) and other birth defects (4) but it is not the only consideration. There is evidence that a number of socio-demographic and lifestyle factors can also influence folate status either positively or negatively. Prior to FA fortification, factors independent of folate, such as adolescence, low socio-economic status, low consumption of folate-rich foods, smoking, alcohol use, and low compliance with FA containing supplements were all associated with an increased risk of having an NTD (51-53). More recent work done in Canada, post-fortification, found positive associations between RBC folate concentrations with the lowest NTD risk and high income, age, the use of FA and/or B_{12}-containing supplements, being born in Canada and high intakes of fruits and vegetables (54). However, a number of factors previously shown to have an impact, such as alcohol use, were not included in the post-fortification work and may still adversely influence NTD-related risk. Further, the work lacks details regarding FA supplementation use which limits the interpretation of the study. Therefore evaluating a comprehensive list of variables shown to be associated with affecting the folate status of healthy WCBY and assessing which variables are significantly associated with RBC folate concentrations known to be protective of NTD as well as including more details about FA supplementation use, such as dose and frequency of use, may further refine our understanding of how much FA is truly required to achieve RBC concentrations associated with NTD reduction. We would theorize that a comprehensive list of variables would better improve our ability to predict if a woman is more likely to have RBC folate concentrations associated with NTD protection.

While FA supplementation is the most common means of improving folate status, less than 1/3 of WCBY use FA-containing supplements (55, 56). Diet then becomes their sole source of folate. While intakes of folate have been recently reported among Canadian WCBY by our research team (56), the specific foods consumed that contribute to reported dietary folate intakes
at the national population level have not been systematically investigated. Further, while folate has been the nutrient receiving most of the attention, there are other nutrients in one carbon (1-C) metabolism, notably vitamins B\textsubscript{6} and B\textsubscript{12} and choline, whose intakes and status are just as crucial for maintaining adequate function of 1-C metabolism. While 1-C metabolism is governed by the intermediary folate metabolites with THF as the central metabolite between the methylation and synthesis pathways (44), choline is the other methyl group donor for 1-C metabolism where it is oxidized to betaine and then contributes a methyl group to homocysteine in the methylation pathway. Vitamin B\textsubscript{12} regulates the conversion of homocysteine to methionine, and vitamin B\textsubscript{6} regulates the conversion of THF to 5,10-methyleneTHF in the synthesis pathway (57). Further, there is a growing body of evidence suggesting that not only folate, but choline, B\textsubscript{12}, and to a lesser extent B\textsubscript{6}, are also associated with NTDs (58). Indeed, it has been noted that the interrelationship between these 1-C vitamins (folate, vitamins B\textsubscript{6} and B\textsubscript{12}, and choline) may actually have a greater impact on methylation patterns and NTD risk than the deficiency or high blood concentrations of any single 1-C nutrient (57).

Dietary intakes of vitamins B\textsubscript{6} and B\textsubscript{12} have been examined at the population level in both Canada and the U.S. and intakes are generally above the estimated average requirement for WCBY (59, 60). Wider spread use of vitamin B\textsubscript{12} in the U.S. food system, due to looser mandatory and voluntary fortification regulations, ensures that overall B\textsubscript{12} intake is higher in the U.S. population than in Canada (59, 60). Data from the Canadian Health Measures Study show that approximately 5\% of WCBY are B\textsubscript{12} deficient (< 148 pmol/L) and approximately 20-25\% have marginal B\textsubscript{12} status (148-221 pmol/L) which could increase their risk of B\textsubscript{12}-mediated NTDs (61). Currently, in both Canada and the U.S. there are no national data looking at the dietary food sources for vitamins B\textsubscript{6} and B\textsubscript{12} and what little information that does exist is based on dated fortification rules (62). Further, there is limited evidence looking at choline intakes across the U.S. population (63) and are no data on choline intakes in the Canadian population. It was found that choline intakes were predominately below the recommendations for the majority of the U.S. population (63). We are interested then, in what foods Canadian WCBY are eating for intake of these crucial nutrients as the majority are not relying on the use of supplements to meet their requirements. Further, attention to the types of foods chosen by women may help address issues with diet quality and vulnerability of intake due to food trends, preferences or food security (64).
2. REVIEW OF THE LITERATURE

2.1. INTRODUCTION TO FOLATE

2.1.1. Description of Folate

Folate is the generic term for a chemically-related and metabolically interconvertable group of compounds involved in one-carbon metabolism. Discovered in the early 20th century as a hemopoietic factor that cured macrocytic anemia, which often occurred in late pregnancy, folate derives its name from the Latin *folium* (65, 66). A water-soluble B-vitamin, folate is involved in amino acid metabolism, the synthesis of purine and thymidine nucleotides and the production of S-adenosylmethionine (SAM), a compound that is involved in the methylation of various biological substrates including DNA, proteins and lipids (67). Structurally, folate is a tripartite molecule comprised of a pteridine ring structure (2-amino-4-hydroxy-pteridine) joined at the C-6 position via a methylene group to p-aminobenzoic acid, and a number of glutamic acid moieties linked by γ-peptide bonds (*Figure 2.1*) (44). Naturally-occurring folates differ in the one carbon substituents at the N-5 and N-10 positions of the pteridine ring and in the length of the polyglutamyl chain ranging from 2 to 9 glutamate residues (67). Intracellularly, folylpolyglutamates improve cellular retention and are the preferred substrate forms with increasing affinity and decreasing $K_m$ values for folate-dependent enzymes with increasing glutamyl chain length whereas monoglutamates are used for extracellular transport (44, 65). Folic acid (FA) or pteroylmonoglutamate is the synthetic and most oxidized form of folate used in fortification and supplementation due to the stability of its structure. Mammals require preformed folate from dietary sources since only microorganisms and plants have the ability to synthesize the pteridine ring of folate (44). Naturally-occurring folates are chemically labile and light sensitive but stability differs depending on their carbon substitutions (44). Food folates are found mainly as 5-methyltetrahydrofolate (5-methylTHF) and to a lesser degree as formyltetrahydrofolate (formylTHF) (65, 67).
Figure 2.1 Structure of Folate

Pteridine ring

$p$-aminobenzoic acid

Glutamic acid or polyglutamate chain

n=2-11

Courtesy of Susanne Aufreiter
2.1.2. Metabolic Functions

Folate mediated one carbon metabolism is a complex series of interconnected pathways with each of the different folate forms having a specific role in intracellular metabolism; the polyglutamyl form of tetrahydrofolate (THF) is the central bioactive compound from which all other folate forms are generated (1, 67) (Figure 2.2). Other nutrients involved in one carbon metabolism include vitamins B$_6$ (required for the conversion of homocysteine to cysteine using 2 vitamin B$_6$ dependent enzymes, and as a cofactor to serine hydroxymethyltransferase in the conversion of THF to 5,10 methyleneTHF); B$_{12}$ (a precursor to methionine synthase which converts homocysteine and betaine to methionine; and choline (methyl donor) (57).

The primary forms of folate found in circulation are pteroylmonoglutamates with 5-methylTHF being the dominant form (44). Intracellular folates are polyglutamated and primarily protein bound, being distributed between the cytosol and mitochondria with low concentrations of free folate forms (68). Up to 50% of cellular folate is found in the mitochondria with the longer folylpolyglutamates concentrated in the mitochondrial folate pool (44). Choline degradation and the synthesis of formate (from serine catabolism), glycine, formylated-methionyl-tRNA, are all generated from folate metabolism in the mitochondria (44, 67). The role of mitochondrial folate metabolism is to generate a supply of 1-carbon units for DNA synthesis and methylation in the cytosol (69).

The one-carbon forms of THF are converted enzymatically in the cytoplasm (67, 70). 10-formylTHF is used in the formation of the purine ring and to synthesize formylated methionyl-tRNA; while 5,10-methyleneTHF is used to convert deoxyuridine monophosphate to deoxythymidine (a pyrimidine) utilizing serine as a carbon source and generating glycine; and finally 5-methylTHF is used in the remethylation of homocysteine to methionine which is then converted to SAM, one of the major methylation cofactors used in over 40 different reactions (67, 70). 5-formylTHF while not a metabolic cofactor, is the most stable form of reduced folate and its polyglutamated forms act as regulators of the enzymes responsible for 1) the conversion of serine and THF to glycine and methyleneTHF generating 1 carbon units used for purine and thymidine synthesis, and 2) the incorporation of formate into the purine ring (71).
Figure 2.2: The one-carbon cycle of folate metabolism. Schema for 1-carbon metabolism with tetrahydrofolate (THF) central to the production of purines and thymidine as well as the methylation pathways involving methionine synthesis. AICARTR aminoimidazolecarboxamide ribotide transferase; Cth,cystathionine ;Cys, Cysteine; dTMP, deoxythymidine monophosphate, dUMP, deoxyuridine monophosphate; DHF dihydrofolate; ;DHFR, dihydrofolate reductase; FA, folic acid; Gly, glycine; Hcy, homocysteine; Met, Methionine; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SAM S-adenosylmethionine, SAH S-adenylhomocysteine; SHMT, serine hydroxymethyltransferase, THF, tetrahydrofolate
The first signs of folate deficiency occur in tissues with rapid cellular turnover and during periods of increased cell division, such as pregnancy, with impaired RBC synthesis resulting in megaloblastic anemia (72). Folate accumulates only in the developing reticulocytes and is a good indicator of folate tissue stores (42, 67, 73). Folate or B\textsubscript{12} deficiency inhibits purine and thymidylate synthesis leading to impaired DNA synthesis from strand breakage and nuclear damage in the developing erythroblasts, producing large immature cells with hyper-segmented neutrophiles and higher rates of cellular apoptosis (73). Supplementing with folic acid (FA) will push the synthesis pathways, resulting in normal erythroblast formation but will not correct neurological symptoms of B\textsubscript{12} deficiency (72, 73).

2.1.3. Digestion, Absorption & Metabolism

During digestion, dietary folate polyglutamates must be converted to the monoglutamate form by glutamate carboxypeptidase II (or \(\gamma\)-glutamyl hydrolase) in the epithelial brush border prior to absorption across the jejunum intestinal wall since only the monoglutamate forms are effectively transported in circulation (42, 67). Naturally occurring folates are primarily found in the reduced polyglutamated forms of methyl- and formylTHF and relative to FA, the fully oxidized, monoglutamate form of folate, have lower bioavailability (the amount actually absorbed compared to the amount ingested) and are dependent on: factors that can affect its release from the food matrix; the overall lability of the folate form; alterations in gastric and intestinal pH; as well as post-absorptive factors such as genetics, folate stores, competing nutrients, and other biological/physiological factors (74, 75). Imperfect deconjugation causes incomplete absorption but evidence suggests that polyglutamyl forms can still exhibit approximately 50-80% bioavailability (75).

Folate is transported via two types of carrier-mediated mechanisms, transmembrane carriers and binding proteins (44). Absorption occurs primarily in the upper jejunum, and less effectively in the distal jejunum and ileum, using saturable proton coupled, pH- and energy-dependent folate carrier proton coupled folate transporter (PCFT) which does not distinguish between FA and naturally occurring folates but at slightly acidic conditions such as pH of 6.5, 5-methylTHF is transported more effectively than FA (44, 76, 77). Reduced folate carrier (RFC)
is also expressed in the intestinal mucosa, but transport into the intestinal mucosal cells is primarily the work of the transmembrane PCFT carrier (44). PCFT is highly expressed in the small intestine but is also expressed in other tissues such as the brain, kidneys, liver and the placenta (44). PCFT is thought to be the primary method of folate transport not only in the gut, but in the liver while transport of folate in the choroid plexus is thought to be a combination of PCFT and folate receptor α (44). PCFT is expressed at lower levels in the colon but the natural pH of the colonic environment makes the extent of absorption from this region unclear (78). A recent study by our lab quantified colonic absorption as approximately one-fifth that of the small intestine, but the increased transit time in the colon made the net absorption much smaller (79). RFC is expressed in adult tissues and is the primary method of transport for folate to cells within systemic circulation under normal conditions (44, 78). RFC is expressed not only in intestinal mucosal cells but in other epithelial cells including the basolateral membrane of the proximal renal tube, and the choroid plexus (78). RFC has a much lower affinity than PCFT for FA but a high affinity for 5-methylTHF and 5-formylTHF and for the antifolates and functions at the more neutral pH of 7.4 (44, 78). It is a saturable, facilitative carrier capable of transporting folate into cells to create high electrochemical-potential differences across the cellular membrane (44, 78).

The second transport mechanism is the folate-binding proteins or receptors. Folates are bound with high affinity to the folate receptor on the cell membrane surface which then allows for unidirectional transport of the complex into the cell (44). There are 3 types of receptors with most tissues expressing the α form. Tissues with the highest expression of receptors include choroid plexus, the kidney proximal tubes, and the placenta (44). These receptor proteins show the highest affinity for FA with 5-methylTHF being lower. Other than the receptor-mediated reabsorption of folate which occurs in the kidney proximal tubes, the role of these receptors in folate transport is not well understood (44). RBC lack the ability to accumulate and transport folate; what folate they do contain is accumulated during erythropoiesis and retained during the lifespan of the cell (44). Normally, plasma does contain a soluble form of the folate receptors in low concentrations but which increase greatly during pregnancy (3, 44).

Once folates are absorbed they enter the portal circulation and are transported to the liver where they are converted back to the polyglutamyl form and will enter into folate metabolism, or be stored (42, 44). The liver retains approximately 10-20% of absorbed folates from the first pass (72). They can also be converted back into their monoglutamate form and released back into
circulation (42). There is a delay in FA absorption since FA, the synthetic form of folate, must be reduced by dihydrofolate reductase (DHFR) first to dihydrofolate and then to THF. This conversion occurs in the liver and to a lesser extent in enterocytes, which means that FA is exported into the hepatic portal vein primarily unaltered (80). Further the rate of conversion activity by DHFR is extremely low in humans (less than 2% of the activity in rats), which can lead to unmetabolized FA appearing in circulation with FA doses of \( \sim 200\mu g \) or greater (80-82). The liver is the primary site of stored folate, containing approximately 50% of body stores which is estimated to be 6-14 mg out of a total body stores of approximately 12-28 mg (42). In plasma, approximately two-thirds of folate is bound to low and high affinity binders, to stabilize the labile monoglutamate folate form, of which approximately 50% are bound to albumin (42). Cellular uptake is done primarily by the reduced folate carriers, a saturable transport system with higher affinity to the reduced folate forms (44). One inside tissue, folates are converted to the polyglutamyl forms by folylpolyglutamate synthase and enter metabolic processes (42).

Folates that are not protein bound in plasma can enter bile where they are usually reabsorbed (in normal healthy individuals) in the small intestine or in the kidney proximal tubules (44). Biliary excretion of folates is variable, up to 100 µg/d, amounts attributable to actual losses from circulation and folate synthesized by the gut microbiome (44). Reduced folates that are not bound to folate-dependent enzymes or binding proteins are subject to catabolism at the C9-N10 bond, an irreversible reaction resulting in the formation of 6-formyltetrahydropterin and p-aminobenzoylglutamate (44, 68). These products undergo excretion in urine (68). Overall rates of whole-body folate turnover are slow in normal conditions with folate pools existing more than 100 days but variations are tissue specific with the liver having much slower turnover which may be due to higher concentrations of folate binding proteins (67, 68). Increased rates of folate catabolism occur with increases in cellular division and folate intake; suggesting a process to maintain intracellular folate concentrations by initiating catabolism due to the increased presence of formyl THF forms or higher intake (44, 68).

2.1.4. Sources & Bioavailability

2.1.4.1. Naturally Occurring Folate
As might be expected, given the derivation of its name from folium, naturally occurring folate is found predominately in leafy green vegetables and other plant foods. Food sources considered high in naturally occurring folate (taking into account overall amount and bioavailability) are leafy greens, pulses, fruit, baking yeast and liver (74, 83). Folate concentrations are higher in plant foods with lower water content, such as legumes, with concentrations ranging upward of 600 µg folate /100g, while green leafy vegetables contain up to 200 µg folate/100g and baking yeast over 1000 µg of folate/100g (83). Meat (except liver, the storage site of folate) and dairy are not good sources of folate. However, processes such as fermentation can improve folate concentrations with cheese containing up to 100 µg of folate per 100g (83).

Overall, folate bioavailability in fruits and vegetables has been estimated to be approximately 60-98%, with equivalent contributions from mono- and polyglutamyl forms (75). The labile nature of natural-occurring folates means that food processing can adversely affect folate content. Heat treatment (such as boiling) causes both oxidative degradation to the folate molecule as well as interconversions of several of the folate forms creating losses from 50-80% in leafy greens (but only 2-10% in legumes) depending on the extent and length of time of processing (83-85). Some of the literature in the area of folate bioavailability from foods has been confounded by underestimation of the folate content in foods and or in biological tissues. The use of different methodologies used for extraction and measurement, using FA as a reference standard rather than the less bioavailable food-based reduced folates, and the use of whole foods versus isolated compounds mean that results on food folate bioavailability are often variable or limited and it is not possible to determine absolute bioavailability (74, 84, 86).

2.1.4.2. Fortification

Mandatory fortification with folic acid was implemented in Canada in 1998 to reduce the risk for neural tube defects (NTD) (12). Cereal grain foods including white wheat flour and pasta labelled enriched (in the U.S.A, rice and corn-meal are also fortified) were fortified with 150 µg FA per 100g and 200 µg FA/100g respectively, which was estimated to increase the median intake of folate by 100 µg across the population (12, 87). Evidence suggests however, that the actual mean contribution to overall intake is closer to 200 µg/d, rather than the estimated
100 µg (75, 87). The higher intake is attributed to manufacturer overages which contribute on average 150% of the amount listed on the Nutrition Facts panel or in the Canadian Nutrient File; rolls and buns had the lowest overages (117%) while ready to eat breakfast cereals had the highest (237%) (56). In a post-fortified United States, breads, rolls and crackers replaced vegetables as the single largest contributor (15 % of total intake) of folate/FA containing foods (88).

To account for the variation in bioavailability from food and fortified food sources dietary folate equivalents or DFEs were introduced in 1998. Since FA does not require release from food matrices, and is already in the oxidized and monoglutamate form, it is considered to be much more bioavailable than naturally occurring forms of folate (74). In general, food folate is considered to be 50% bioavailable while FA is considered to be approximately 85% when consumed with food (as in fortified food sources) or 100% when consumed alone. One DFE is equal to 1 µg dietary folate and 0.6 of FA supplement, so FA is 1.7 times as bioavailable as naturally occurring folates (74).

Data from the NHANES III and the NHANES 1999-2000 compared food sources of folate pre and post fortification (88) (Table 2.1). There was a shift to fortified foods post fortification with breads, rolls and breakfast cereals contributing ~28% of total folate intake. A recent study by our group in Toronto looking at the dietary intakes of a small group of pregnant and lactating women found that orange juice was the top food source (~11%) while FA fortified foods (including pasta) contributed about 31% of total folate intake (89). However, at this time there is little information about folate dietary sources for Canadian women of reproductive age at the national level.
Table 2.1: Comparison of the top 5 food sources for folate pre- and post-fortification in the US population

<table>
<thead>
<tr>
<th>Rank</th>
<th>Pre-Fortification</th>
<th>Post-Fortification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Description</td>
<td>% Total folate</td>
</tr>
<tr>
<td>1</td>
<td>Vegetables</td>
<td>19.4</td>
</tr>
<tr>
<td>2</td>
<td>Breakfast cereals</td>
<td>17.7</td>
</tr>
<tr>
<td>3</td>
<td>Breads, rolls, crackers</td>
<td>9.1</td>
</tr>
<tr>
<td>4</td>
<td>Beans, peanuts</td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td>Fruit juice</td>
<td>8.7</td>
</tr>
</tbody>
</table>

1Total folate defined as mg/d. Information was derived from (88).

2.1.5. Measuring Serum and RBC Folate Concentrations

The primary indicators of folate status are serum and RBC folate concentrations; homocysteine is sometimes used as a functional test for folate deficiency; however it is nonspecific because both folate and B₁₂ are required as cofactors in the remethylation of homocysteine to methionine. Serum folate concentrations will fluctuate with recent folate intakes both increasing and decreasing, and can be adversely affected by age, alcohol intakes, and medications such as antibiotics, pregnancy (without the intervention of fortification or supplement intake) certain anticancer and anticonvulsant drugs, smoking and liver damage. RBC folate concentrations are a measure of long term folate status as they are an indicator of tissue stores. Folate is incorporated into RBC during erythropoiesis in bone marrow. RBC folate is not influenced by recent dietary fluctuations in intake but can be adversely influenced by smoking, pregnancy (without additional FA), B₁₂ and/or iron deficiency. Traditionally, both serum and RBC folate concentrations have been measured using the microbiological method (still considered the gold standard) and competitive folate protein binding assays such as the Bio-Rad. More recently, mass spectrometry methods are being used specifically for the measurement of folate derivatives, these methods are not yet perfected but are capable of measuring all folate forms plus varying glutamyl tail lengths. More detailed discussion of measurement methodology is provided in Section 2.3.1.
2.1.6. Folate Status of the North American Population

Normal folate status is defined by either serum or RBC concentrations. Values for normal concentrations are shown in Table 2.2.

Table 2.2. Folate concentrations in serum and red blood cells reported in the literature

<table>
<thead>
<tr>
<th>Serum/plasma folate concentrations nmol/L (ng/ml)</th>
<th>Red blood cell folate concentrations nmol/L (ng/ml)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 7 (&lt; 3)</td>
<td>&lt; 305 (&lt; 135)</td>
<td>Institute of Medicine (42)</td>
</tr>
<tr>
<td>&lt;10 (&lt;4)</td>
<td>&lt; 340 (&lt; 151)</td>
<td>WHO (15)</td>
</tr>
</tbody>
</table>

Deficiency of folate concentrations leads to increases in homocysteine concentrations, megaloblastic changes in bone marrow and tissues with high turnover leading to macrocytic anemia if not corrected (42). During pregnancy, deficiency has been demonstrated to increase the risk for megaloblastic anemia, cleft lip/palate, congenital heart defects and neural tube defects (NTD) (42, 92). It is because of this relationship with NTDs that both Canada and the United States mandated FA fortification of cereal grain products (labelled as enriched) in 1998. Prior to fortification, data from NHANES III (1988-94) showed that approximately 38% of women had RBC folate concentrations below 340 nmol/L, with those of childbearing years having mean RBC concentrations of 686 nmol/L and 14 nmol/L for serum folate (15, 17, 88). The impact of FA fortification on folate status has been significant across North America with the prevalence of risk of low RBC folate (< 340 nmol/L) in the United States dropping to approximately 5% in 2004 and less than 1% by 2010 while geometric mean RBC concentrations in the general population rose to 1060 nmol/L (15, 17). At the same time, the estimated number of individuals with high serum folate concentrations of > 20 ng/ml (45 nmol/L) increased in children (5 to 42%) and older adults (7 to 38%) from pre to post fortification (17). The high serum cut-off is based on the estimated 97% ile for serum folate concentrations in the U.S. population prior to fortification (17). Overall, the change in RBC concentrations increased by 150-180% early post-fortification and then declined slightly as folate intake decreased both from reductions in over fortification and alterations in dietary patterns (15, 93). In Canada, increases of 130% were seen
in RBC folate concentrations pre- to post-folic acid fortification in a small population study in Newfoundland. Concurrently, in the same study the rates for NTD fell 78% (94). A recent national study found that < 1% of Canadians had RBC folate concentrations below 305 nmol/L but 40% showed high folate concentrations (defined as > 1360 nmol/L) (16). The high cut-off for RBC folate was based on the same U.S. NHANES data used to determine the serum folate cut-off in Pfeiffer et al (17); however a slightly more conservative approach was taken and the 97% ile of the U.S. population’s RBC folate (> 1360 nmol/L) was determined as the cut-off for high RBC folate (16).

2.1.7. Requirements

2.1.7.1. General Population

Requirements for folate are based on maintaining normal one-carbon transfer reactions and use RBC folate as an indicator of folate status (1). Folate, incorporated into RBCs during erythropoiesis is considered a good indicator of tissue folate concentrations and while plasma folate can be influenced by recent dietary intake (42). Plasma homocysteine may be used in conjunction with serum or RBC folate concentrations since values increase during folate deficiency due to reduced remethylation of homocysteine to methionine. However, homocysteine is not a specific marker of folate deficiency since it is influenced by deficiencies of vitamin B₆ or B₁₂, age, gender, and genetic polymorphisms (42, 44). The estimated average requirement (EAR) for folate is based on meeting the needs of 50% of the population (42). The information to derive the folate requirements for adults is based on quantitative studies that demonstrated the amount of folate necessary to maintain normal RBC folate concentrations (>305 nmol/L) to be 200 - 400 µg/d DFE (42, 74). Due to limited evidence, only an AI exists for infants less than 6 months of age, while for infants 7 to 12 months the EAR is extrapolated from the adult values (42). For adults over 19, for both men and women, the EAR is set to 320 µg/d DFE and 10% coefficient of variation to set the RDA to 400 µg/d DFE (42). A Tolerable Upper Limit (UL) was based on synthetic folic acid only and is set at 1000 µg/day, as intakes above this may mask B₁₂ deficiency, creating delays in diagnosis, and exacerbating the neurological damage from vitamin B₁₂ deficiency (42).
2.1.7.2. Reproduction

Folate requirements increase significantly during pregnancy to meet the demands for DNA/RNA synthesis and increased methylation of proteins to support the growth of fetal and uteroplacental tissues, maternal red cell mass as well as placental folate transfer (3). Early recommendations were based on maintaining RBC folate concentrations (> 305 nmol/L) and preventing folate deficiency during the latter part of pregnancy (3). However, it has been shown that periconceptual folic acid supplementation (8, 95) and fortification of the food supply (5, 6) have also been shown to reduce the risk of pregnancies with NTD. Yet, despite this link, the recommendations for women during pregnancy are based on maintaining RBC concentrations, a reflection of tissue stores, as the criterion for adequacy, and not prevention of NTD (42, 96). Evidence suggested that 450 µg/d (or 200-300 µg/d of FA with the remainder from dietary folate) was needed to in order to maintain normal RBC folate concentrations and prevent folate deficiency during pregnancy (42). Based on this, an EAR was set to 520 µg/d DFE while the RDA was set to 120% of the EAR (and agreed with the findings in the research) so that a RDA of 600µg/d DFE was sufficient to meet the needs of 97 to 98% of pregnant women (42). Periconceptual guidelines are suggested to reduce the risk of NTD; for women capable of becoming pregnant, Health Canada recommends 400 µg folic acid from supplements, in addition to a varied diet, in order to reduce the risk for NTD, for 3 months prior to pregnancy and to continue for the duration of the pregnancy (38, 97).

2.2. FOLATE AND REPRODUCTION

2.2.1. Function

Folate requirements increase during pregnancy. The primary role of one carbon metabolism is to provide one carbon units to promote DNA/RNA, protein and phospholipid synthesis and methylation of DNA, proteins and histones; the increased metabolic demand by developing maternal and fetal tissue means optimal folate status is crucial. Physiological changes during pregnancy, in the absence of supplementation, include a decrease in maternal
serum and RBC folate concentrations up to 10 nmol/L not only from this rapid growth but also due to hemodilution, increased folate catabolism as well as fetal transfer of folates via the placenta during the last month of gestation (3, 92, 98, 99). Without supplementation or fortification, there is an increased risk of megaloblastic anemia or placental abruption (3, 100). As a result of hemodilution, serum folate is not a good measure of folate status during pregnancy (42, 96).

2.2.2. Metabolic Changes

While the evidence is inconsistent, pregnant women do demonstrate rapid folate clearance and alterations to catabolism and excretion of folate which is thought to be related to increased demands for folate (3). Caudill et al (2) found that urinary folate catabolism does not increase during the second trimester but that pregnant women may be more efficient at conserving folate than non-pregnant women (2). While Gregory et al (43) found marginal kinetic changes in metabolism, turnover and excretion in folate replete pregnant women versus non-pregnant controls, the authors postulated that the relatively small kinetic changes seen in their study would be more substantive in those with marginal folate intake.

Homocysteine concentrations during pregnancy normally decline in the first trimester, reaching their nadir towards the end of the second semester, and subsequently rise during the third and may be related to hormonal changes (3, 101). Elevated homocysteine concentrations are associated with pre-eclampsia and gestational hypertension but direct causality has yet to be established (3, 102). Fetal transfer of folate occurs in the latter half of pregnancy. The placenta is rich in folate-binding proteins (FR-α) and in a two-step process, binds circulating maternal 5-methylTHF, primarily from dietary intake, and concentrate intervillous blood folate, to ~3 times maternal concentrations, allowing folate transfer to the fetus in a downhill concentration gradient (3, 98, 103). RBC folate concentrations in the placenta remain similar to maternal concentrations, suggesting that the substrate for fetal transfer is based on maternal dietary intake of folate and FA rather than placental folate stores; therefore, declines in maternal intake could potentially influence overall amount of folate transferred to fetus or decrease maternal concentrations (98, 103).
2.2.3. Folate and Birth Defects

2.2.3.1. Neural Tube Defects

It has been estimated that the number of NTD-affected newborns in the early 1990s (prior to the push for FA fortification worldwide) was a minimum of 300,000 each year or a prevalence rate of 0.5 to 60/10,000 births with regional and population specific variations (92, 104). NTD are the most frequent congenital anomalies caused by improper closure of the neural tube (the precursor to the central nervous system) between days 22 to 27 after fertilization, and before most women know they are pregnant. The most common forms are anencephaly which is failure of closure at the cranial end of the tube, and spina bifida which is a failure of closure at the caudal end (105). Anencephaly is characterized by the absence of the cranial vault and brain, and infants with this defect are either stillborn or die shortly after birth; while spina bifida manifests as exposure and/or herniation of the base of the spinal cord resulting in varying degrees of physical and mental disabilities (104, 105). NTD are multifactorial and while the mechanism for folate-related NTD remains unclear, it is generally understood that any dysregulation affecting one carbon metabolism increases the risk of NTD (104). Alterations in folate metabolism can occur through a number of factors including: reduced folate status; single nucleotide polymorphisms in genes affecting one carbon metabolism; and, deficiencies in nutrients that affect folate status (104, 105). Potential mechanisms that can affect neural tube closure stemming from these factors include: homocysteine accumulation; aberrant DNA methylation; and, impairments in nucleotide biosynthesis (104, 106). High concentrations of homocysteine are cytotoxic and potentially teratogenic with elevated maternal homocysteine concentrations linked with increased NTD risk; however, causality has not been demonstrated (3, 104). Aberrations in methylation are also considered a potential mechanism of NTD since epigenetic regulation of cell migration or cellular differentiation could be critical for embryonic development and proper neural tube closure, given that embryonic neural tube and neural crest cells can double in as little as 5 hours (103, 104). Impaired biosynthesis could affect the provision of nucleotides to sustain rapid cellular proliferation required in the neurulating embryo (104). Recently, Leung et al. demonstrated that concurrent administration of thymidine and adenine or guanosine monophosphate produced up to an 85% lower frequency of spina bifida
and exencephaly frequency in mouse models suggesting that both pyrimidine and purine precursors initiate cellular proliferation and stimulate neural tube closure (107).

The link between folate and NTD protection was suggested as early as the mid 1960s by Hibbard (100), who linked excessive formiminoglutamic acid (an intermediary in the breakdown of histidine to glutamic acid which requires THF for the conversion) in pregnant women to abnormal outcomes such as abruption placentae and fetal malformation. However, it wasn’t until later, that two trials provided strong evidence that folic acid prevented the reoccurrence of NTD at 4 mg FA and the first occurrence of NTD at 800 µg FA with a 70-72% reduction in NTD (8, 95). Based on the evidence from these and other studies, FA doses from 360 to 4000 µg before conception and during the first trimester are associated with protection against NTD (108). Higher folate status, as measured by RBC folate concentrations, is also associated with a reduction in NTD risk. Daly et al. (9) found an 8-fold reduction in the incidence of NTD between women in the lowest distribution RBC folate concentrations (<340 nmol/L) compared to those in the highest distribution (> 906 nmol/L). The authors found a continuous dose-response relationship between folate status and NTD protection even with RBC concentrations above the lower limit of the ‘normal’ range at 317 nmol/L (9). These results have come under question as the Celtic population, on whom the study population are based, have a higher incidence of NTD (109) and a higher proportion of the variant gene for the C677T methylenetetrahydrofolate reductase (MTHFR) genotype, effectively doubling the odds of having a NTD affected pregnancy (110). Because of this, it has been suggested that the Celtic population may be genetically predisposed to require higher RBC folate concentrations reduce the risk of NTD. It is known that NTD do occur in women with RBC folate concentrations above 340 nmol/L, suggesting that folate deficiency alone is not the primary mechanism of NTD and that there may be a genetic component (9, 104).

At this time there is no definitive lower boundary above which maximal folate-related NTD protection occurs for women of childbearing years, but the lowest value of the range associated with the highest protection (906 nmol/L) in Daly et al. (9) does suggest an appropriate target at this time until further proof refines the definition of ‘optimal folate status’. A recent study provided further reinforcement of the range of approximately 1000 nmol/L RBC concentration as a threshold for substantive NTD risk reduction (111) suggesting that the results of Daly et al. (9) can be utilized in other populations. The associations of higher RBC folate concentrations and
FA supplementation reducing risk of NTD led first to recommendations for supplementation of 400 µg/d periconceptually; then, because the majority of pregnancies are unplanned and at any given time only ~ 25% of women of childbearing years are taking a FA supplement, to recommend fortification of food staples (112-114).

Fortification was mandated in 1998 in both Canada and the United States with the estimations of reducing NTD rates by 20-50 percent (112). Data from the United States estimated a low 26% reduction for all cases up to a 41% reduction in spina bifida depending on whether data included prenatally diagnosed and terminated cases (5, 14), while Canadian data reported a 46% reduction using data that included live births, still births, and terminations of pregnancies due to fetal anomalies (6, 94). In Canada, the overall incidence of NTD decreased from 1.58/1000 live births pre-fortification to 0.86/1000 post-fortification, with a clear east-to-west gradient in the effect of the magnitude of rate reduction (6). For example, in the province of Newfoundland (pre-fortification rates of 3.40/1000) NTD rates declined by 78% to 0.96/1000 after the implementation of folic acid fortification (94).

While fortification has been a success story for NTD risk reduction, there is growing evidence that FA supplementation will not completely eliminate all NTD, but rather a ‘floor effect’ exists at about 0.5/1000 births (58, 115). This floor effect may be the reason that some studies are finding no additional risk reduction with periconceptional FA supplement use if dietary intake of natural and fortified foods is high (116, 117).

2.2.3.2. Defects Including Cleft Lip/Palate and Other Issues

While the evidence linking FA with NTD is strong, the evidence for the protective effects of FA against other birth defects is inconsistent. Both the British Medical Research Council (95) and Czeizel & Dudas (8) examined the relationships between FA supplementation and other birth defects, but found no significant difference between treatment groups and controls for cleft lip or palate, congenital heart defects or other birth defects (108). A 2004 review of the literature (17 studies) suggested that a daily multivitamin could prevent up to 5,200 birth defects worldwide daily, but the majority of these defects consisted of NTD and congenital heart malformations. The authors of this review suggested the evidence for facial clefts was
inconsistent (4). A recent meta-analysis (5 cohort and 12 case/control studies between 1958-2003) found a reduced risk for all clefts (0.55, 95% CI: 0.32, 0.95) when evidence was combined, although the authors suggest that they could not eliminate all confounding factors (7). A population study in Norway (no mandated FA fortification) found the highest risk reduction (0.36, 0.17 to 0.77) in the rate of facial cleft with women who used FA supplements (≥400µg/d) plus a folate rich diet, suggesting some additional protective effect from other nutritional factors in a healthy diet (118). Maternal occupation is also linked to risk of various birth defects but FA supplementation may help to mitigate the risk; a recent U.S. study found an interactive effect between maternal occupation and FA supplementation with a lower risk of facial clefts in female janitors and preschool teachers with FA supplement use prior to conception and through the first month of pregnancy in cases versus controls (119).

Other adverse pregnancy outcomes are reduced with improved folate status. Higher folate concentrations and/or periconceptual use of FA supplements were associated with a decreased risk of intrauterine growth restriction, improved birth weight and lower risk of spontaneous abortion; however, rates of twinning were not increased in normal pregnancies (120-124). Other evidence (case/control and randomized control trials) found that the risk for cardiac defects were reduced by approximately 24-50% with the use of pre-natal multivitamins containing FA, although the evidence suggests that ventricular septal defects may benefit the most (122).

2.2.4. High Maternal Intakes of Folic Acid

2.2.4.1. Current Knowledge

The concept of fetal programming refers to epigenetic alterations occurring specifically during the gestational period. Epigenetics is defined as heritable modifications of gene expression without altering the DNA sequence; such modifications are dynamic throughout the lifespan allowing for flexibility in phenotypes (57, 106, 125). Epigenetic coding includes DNA methylation, chromatin remodelling, covalent histone modifications, and regulation of non-coding RNA and microRNA, all of which alter gene expression and function (57, 126). Epigenetic modifications are influenced by both internal and external environmental factors in a dose dependent manner and are potentially reversible by both dietary and pharmacologic
interventions (106, 126). DNA methylation is currently the most studied form of epigenetic control and is an important mechanism in epigenetic regulation of gene expression, the maintenance of DNA integrity, in the organization of chromatin and the development of mutations (57). In normal cells, methylation of cytosine is common throughout the genome and commonly occurs at cytosine-guanosine sequences (CpG) where up to 80% of all CpG dinucleotides in human DNA are methylated (126). This provides a mechanism of epigenetic regulation through subsequent cell generations for stable transmission of cellular information (127, 128). Outside of these regions are dense, unmethylated regions called CpG islands, which are usually correlated with promoter and other regulatory regions of genes, and are predominately unmethylated (127). Methylation of these CpG islands can suppress or silence the corresponding gene (125, 126). DNA methylation relies on SAM as the methyl donor and availability of SAM is directly influenced by the dietary intake of the methyl nutrients choline, betaine, methionine, folate, and B12 (57, 106, 125). Therefore any perturbations in dietary intake can alter DNA and histone methylation, via SAM and SAH production, thereby affecting gene expression (106, 125).

The epigenome is very susceptible to dysregulation during the gestational period in part due to the high DNA synthesis rate and the dynamic state of DNA methylation during embryogenesis (127, 128). There are several periods of significant changes in DNA methylation during embryogenesis and this includes gametogenesis, the fertilization period, and early development (127, 128). Epigenetic modifications undergo erasure and remodelling during cellular differentiation and development to generate tissue-specific methylation patterns (127, 129). Genomic imprinting occurs during gametogenesis where the DNA of the male or female gametes acquire differential parent-of-origin DNA methylation patterns where either the paternal or maternal inherited allele is expressed or silenced depending on the imprinted gene (127, 128). After fertilization, paternal genome methylation patterns are actively demethylated prior to cell division while the maternal genome patterns are demethylated slowly during the blastocyst stage except in areas of genomic imprinting, heterochromatin around centromeres and retroviral elements (128). After implantation and the beginnings of differentiation, de novo DNA methylation occurs effectively reprogramming to establish the tissue-specific DNA methylation patterns found in fully differentiated adult somatic cells (127-129). The active reprogramming of DNA methylation patterns during gametogenesis and embryogenesis can create periods of
increased susceptibility for environmental influences to alter the normal process of establishing DNA methylation patterns (127). It is possible then, that dysregulation in the methylation pathway during pregnancy may have permanent effects on the fetus by altering disease phenotypes (57, 125, 126).

There is good evidence that alterations in maternal dietary 1-C nutrient intake can influence DNA methylation patterns and phenotype in offspring. Studies using mice clearly show the impact of alterations in 1-C nutrient intake on DNA methylation patterns by altering phenotypic coat colour on the offspring through increased CpG methylation of the promoter region of the agouti gene and by reducing the proportion of mice with kinked tails by higher methylation of the AxinFused gene (27, 130). However, the results are not consistent. A study that fed Wistar rats a high vitamin diet during gestation but weaned rats to a regular vitamin diet showed some effect on hypothalamic gene expression but no difference in global DNA methylation (29). Further, males from the high vitamin diet weaned to the regular vitamin diet showed higher levels of food intake, more weight gain and altered glucose tolerance (29). Similar results were seen in sheep where maternal periconceptional folate and vitamin B₁₂ restriction resulted in altered methylation patterns in 4% of the CpG islands and male offspring demonstrated increased adiposity and insulin resistance (131).

Limited human studies provide further evidence of the effect of FA supplementation during gestation on epigenetic effects in offspring. In an observational study conducted in the Netherlands, periconceptional maternal intakes of 400µg/d significantly increased the methylation of the insulin-like growth factor-2 (IGF2) gene by 4.5% in children at 17 months of age with a significant inverse association of -1.7% between methylation and birthweight (31). Evidence from the Newborn Epigenetic Study evaluated two differentially methylated regions known to regulate IGF2 expression and found that maternal use of FA supplementation prior to and during gestation was associated with significant decreases in methylation in the second differentially methylated region, but not the first, in FA supplement users compared to non-users (132). However, it is not yet determined if alterations in DNA methylation have any long term impact on human health.

**2.2.4.2. Potential Adverse Effects in Offspring from High Maternal Intakes of Folic Acid**
While the discussion about dysregulation in one carbon metabolism has been focused on deficiency of critical nutrients, there is definitely a concern associated with high intakes of FA as well. With the combined effect of fortification and regular FA supplementation, the phenomenon of folate concentrations well above the average physiological range are increasingly more common and raise the theoretical potential for adverse effects. As mentioned earlier, because of the slow conversion rate of DHR in the liver, unmetabolized FA is present in the serum of most individuals consuming folic acid fortified foods or folic acid containing supplements. There is concern about the presence of unmetabolized FA in cord blood of newborn infants suggesting that FA is capable of crossing the placenta even at intakes from fortified foods (25, 82). There is a growing body of epidemiologic evidence suggesting that FA supplementation and/or high maternal folate status during gestation may reduce some (133, 134) but not all (135) pediatric cancers, and autism (136, 137); it may or may not increase respiratory issues and allergy symptoms (33-35); and depending on B12 status, it may or may not affect central adiposity and insulin resistance in children (36, 37). There is some evidence that FA supplementation during pregnancy can influence phenotype frequency within the population (32) and that alterations in methylation of the placenta and IGF-2 (30, 31) can occur but what outcomes will occur from these phenomenon have yet to be determined. The inconsistencies in the current body of evidence limit our ability to determine the true nature of the relationship between possible long-term adverse outcomes and folate status. Further, it is currently unknown if the potential for risk is related directly to FA intake or high maternal folate status, or whether there is an interaction with other one-carbon nutrients or genetic variations that influence risk outcomes. More work is needed to clarify the relationship.

2.3. DETERMINATION OF FOLATE STATUS

2.3.1.1. Microbiological Assay

The microbiological assay has been the traditional method used to measure folate concentrations in both serum and RBC. A number of different strains of bacteria have been used in the past but the most common strain is Lactobacillus casei because growth response is equivalent for all monoglutamate forms of folate (91, 138). L. casei will also respond to di- and tri-glutamate forms, but hydrolysis of the long chain folylpolyglutamates is necessary for more
precise measurement (91, 138). More recent strains are chloramphenicol resistant (NCIB 10463) and eliminated the need for sterilization or aseptic additions which could potentially destroy heat sensitive folates (138). L. rhamnosus (formerly known as L casei) is in current use and is specific to the biologically active folates and is non-reactive to the inactive folate isomers, folate precursors or the breakdown products of folate (139). Naturally occurring folates are very labile and can readily convert to other forms or undergo oxidation (140, 141) so thiols or antioxidants (such as ascorbic acid) are used to stabilize blood samples prior to analysis (141) while subdued or yellow light can also help to limit the degradation of folate in the samples (140). The diversity of the different biological forms of folate (compounded by the various lengths of the glutamyl side chains) can complicate analysis of naturally occurring folate (140). While folate forms in serum are in the monoglutamate form and only require stabilization to prevent degradation; the forms in whole blood require hydrolysis to the tri-glutamyl forms or smaller (91, 138). The application of heat and variations in pH are used, as well as a hydrolase (either endogenous as in whole blood or exogenous, such as rat plasma) depending on the biological matter the folate is to be extracted from, in order to hydrolyze the poly-\(\gamma\)-glutamyl chain (138). The sensitivity and specificity of the assay only requires small sample volumes, while the introduction of 96-well microtiter plates allowed for increased throughput and efficiency both in sample preparation, and the absorbance reading of the bacterial growth in a microtiter plate reader (138, 142). Different synthetic folate calibrators (for comparison of test results) can have slightly different effects in the results of the microbial assay. A recent study compared variations in methodologies for the assay and found that using FA for the calibrator resulted in ~25\% higher readings than using 5-methylTHF as the calibrator (143). The 5-methylTHF is recommended for accuracy as a calibrator for the microbiological assay as the majority of folate in blood is in the 5-methylTHF form while FA is not naturally occurring in serum/plasma after a large bolus from food or supplements (143).

The lysed whole blood samples are incubated at room temperature or 37\(^\circ\)C to allow endogenous plasma folate conjugase to hydrolyze the poly-\(\gamma\)-glutamyl chain. Whole blood is diluted with an ascorbic acid solution (0.5% wt:vol) and incubated at 37\(^\circ\)C for 30 minutes according to Molloy and Scott (142). Prepared samples are diluted by a factor of two (or higher for folate fortified populations) and then aliquoted into the 96 well plate in two different concentrations to register at different points on the calibration curve (142). A 96-well microplate
is prepared and inoculated with the test microorganism and incubated. Turbidity is used to measure the bacterial growth after incubation using a microplate reader (142). The reproducibility of the assay is determined using dilutions of either a serum/plasma certified standard or a whole-blood certified standard each time the test is performed depending on the type of biological sample analyzed. The method is sensitive and relatively inexpensive; however it can be prone to contamination from extraneous folate or microbial source (140).

2.3.1.2. **Liquid Chromatography and Tandem Mass Spectrometry**

A more recent development in measuring folate and folate forms is the use of the LC-MS/MS. This method has the potential advantage as it allows for measurement of individual folate forms and minor derivatives, such as FA, in both serum and RBC (91). The inherent drawbacks would be a potential for increased errors from summing all the parts to derive the whole and incomplete hydrolysis of the polyglutamated forms could potentially reduce how much is recovered and calculated (91). There is also the issue of potential interconversions occurring between species. The microbiological assay counts all forms and even some di- and tri-glutamate forms but interconversions could lead to loss of some forms or artificially raise amounts of others depending on how samples are prepared and handled during measurement of individual forms in the LC-MS/MS method. It has been shown that the LC-MS/MS method used by Pfeiffer et al. yields almost complete recovery of all the folate species, as compared to the stable-isotope internal standards used, and shows good correlation with the microbiological assay (144).

LC-MS/MS analyses use an application of current solid-phase extraction and chromatography techniques to measure folate and the mass spectrometer is used in the multiple reaction monitoring mode for the highest selectivity (144). Labelled folate analogs are used to measure recovery of folate forms in samples. Work is done under gold-fluorescent light to reduce interconversions from UV light or bright light exposure. Ascorbic acid is used in buffer in order to protect the labile folate forms from oxidation during processing. LC-MS/MS use electrospray ionization in the positive mode, as it is much more sensitive that the negative ion mode for 5-methylTHF (144). More recent changes in tandem mass spectrometers technology and changes in methodological approaches have increased the measurement sensitivity, thereby lowering the limits of detection, improving the throughput, reducing the amount of sample
required, and the ability to separate the measurement of 5-formylTHF from an oxidative product MeFox (a pyrazino-s-triazine derivative), both isobaric compounds (145, 146). The first thesis study entitled “Neither Folic Acid Supplementation nor Pregnancy Affects the Distribution of Folate Forms in the Red Blood Cells of Women” utilizes this method and is the first to report MeFox in a clinical study population.

2.3.2. Methodology Issues

2.3.2.1. Comparability Between Different Methodologies

One of the main concerns about measuring RBC folate is the comparability of the several different methods used for measurement. While easier to use and allowing for high throughput, comparison shows the now retired Bio-Rad immunoassay to be approximately 29% lower for serum and up to 45% lower for RBC folate (147). The most likely reason for this disparity may be the under recovery of 5-methylTHF with the Bio-Rad compared to the microbiological assay (147). Other immunoassays are used clinically; however, the values they produce relative to the microbiological assay are less well characterized (148). Most modern immunoassays use chemiluminescence as detection; they have the advantage of being insensitive to antibiotics and can handle high throughput (139). However, they are subject to the limitations of folate binding proteins, and a limited measuring dynamic range making it difficult to maintain accuracy with the higher folate concentrations seen in fortified countries such as Canada and the U.S. (139). Comparison with the microbiological assay shows differences, with underestimations in serum folate and overestimations in RBC folate, limiting inter-laboratory comparisons without careful evaluation (149).

The more recent LC-MS/MS method shows excellent correlation with the microbiological assay for serum folates (r >0.98) but there are still some issues in terms with RBC folate measurement with the results of the LC-MS/MS being approximately 25% higher in comparison to the microbiological assay (147). This may be due to residual diglutamyl folates still present in the hydrolyzed hemolysate (147). There can also be some variability within the microbiological assay itself either through the use of different bacterial strains or calibrators (e.g. FA, 5-methylTHF or 5-formylTHF). 5-methylTHF will produce more accurate results but the
results for serum and RBC folate are approximately 25% lower than when FA is used as the calibration tool in the assay (143).

2.3.2.2. Interconversion of Forms

The pH-related interconversion of some folate forms during analysis is considered a major obstacle for folate identification methods (140). Some naturally-occurring forms of folate are very labile and can easily and rapidly be converted, especially when exposed to light, heat or even air for prolonged periods (141). 10-FormylTHF, 5-formylTHF and 5-formiminoTHF can be converted to 5,10-methenylTHF at acidic pH, with 10-formylTHF converting within minutes (140, 144). The rate of conversion can be slowed by the choice of extraction buffer; however, the rate of interconversions may be faster than the time required for maximum hydrolysis (140). At physiologic pH (~7.4), 5,10-methyleneTHF will disassociate to formaldehyde and THF; thus extractions in this pH range could deplete concentrations of 5,10-methyleneTHF and overestimate THF concentrations (140). Samples heated in ascorbate-containing buffers can also cause some folate interconversion which can result in a loss of THF; the samples can be stabilized using 0.2M mercaptoethanol (140). The use of 1% ascorbic acid solution at 0°C was found to afford better protection than mercaptoethanol with little conversion (141). The pH used in the LC-MS/MS method may cause some interconversion between 10-formylTHF, 5,10-methenylTHF and 5-formylTHF which could potentially affect results of folate form measurements (147).

2.3.3. Folate Form Distributions in the Population

2.3.3.1. Genetic Variability

Common polymorphisms of folate-dependent enzymes, specifically those in the (MTHFR) pathway such as C677T MTHFR and A1298C MTHFR, are known to influence the distribution of folate forms. MTHFR is the enzyme responsible for the conversion of 5-10-methyleneTHF to 5-methylTHF (see Figure 2.2). While both polymorphisms are studied, the C677T MTHFR polymorphism is thought to have a greater impact on enzyme activity and in low
folate environments is associated with elevated homocysteine levels in those who are homozygous for this allele (150). Approximately 10-15% of the North American Caucasian population is homozygous for the C677T MTHFR allele (151). Bagley and Selhub (151), Pfeiffer et al (144), and Fazili et al (152) have shown an altered distribution of erythrocyte folates with the presence of formylated THF polyglutamates and a decrease in the level of 5-methyl THF in individuals who are homozygous for the C677T MTHFR allele compared to wild-type (C/C). Reduced efficiency with the C677T MTHFR polymorphism, can preferentially drive folate pathways away from the methylation cycle towards thymidylate and purine synthesis, leading to elevated levels of homocysteine and reduced production of s-adenosylmethionine (SAM), a compound critical to over 40 methylation reactions in the body (153, 154). Observations in some, but not all studies, have shown the homozygous variant of C677T MTHFR gene in combination with low folate intake to be associated with increased risk of NTDs, Down syndrome, cleft palate/lip and certain complications in pregnancy (3, 155).

2.3.3.2. General Population

There have been several papers examining folate forms in both the general population and a few in female populations, primarily comparing the effect of ethnicity. The majority of these studies compared only serum values and did not examine RBC folate forms. The predominant form of folate found in serum, under fasting conditions, is 5-methylTHF monoglutamate; however, due to the saturation capacity of the cells during absorption, unmetabolized folic acid can appear in circulation among those consuming folic acid fortified foods or supplemental folic acid (76, 82). In studies examining serum folate forms in the general population, the proportion of 5-methylTHF of total folate in serum ranged from 87 to 100%, with 0 to 11% as THF (144, 156). Studies reporting proportion of RBC folate forms in comparison to total RBC folate show greater variability which is not explained by genetic variance alone. Pfeiffer et al (144) reported that those who were wild-type and heterozygous for C677T MTHFR had RBC proportions of 5-methylTHF (90%) and 5-formylTHF (10%), with no THF reported, while those who were homozygous also had significant amounts of 5,10-methenylTHF (10%) and THF (26%). Mitchell et al (157) reported RBC folate proportions of 92% for 5-methylTHF, 7% for THF and 1% for 5,10-methenylTHF in premenopausal women. Friso et al (154) found that RBC folate
concentrations did not appear to influence RBC folate proportions, but there was an effect from the C677T MTHFR polymorphism, with the percent of RBC methylTHF in wild-types (~99%) and homozygotes (~68%) being significantly different. Ethnicity seems to also influence genotypes and RBC folate proportions, with African-Americans having higher occurrences (~70%) of the wild-type and 0% for the homozygous variant for C677T MTHFR genotype, with RBC proportions of 5-methylTHF (98%), THF (1.9%) and very limited occurrence of detectable 5, 10-methenylTHF (155). On the other hand, Caucasian women, who were ~31% wild-type and 19% homozygous, had approximately 87% 5-methylTHF, 9.9% THF with the majority (62%) having detectable concentrations of 5.10-methenylTHF (155, 158). This suggests that ethnicity and the variation of the frequency for the alleles of the C677T MTHFR in the ethnic population can influence the percent distribution of the folate forms. Fazili (159) reported that those who were wild-type and heterozygous for C677T MTHFR had RBC proportions of 5-methylTHF (90%) and 5-formylTHF (10%) with no THF reported. This was altered in those who were homozygous, with concentrations of both 5,10-methenylTHF (10%) and THF (26%) found (159). A later study by the group reported the presence of THF (13-15%) in both American and European RBC folate samples (including wild-type and heterozygous individuals for the C677T MTHFR genotype) and higher ratios for THF (up to 77%) for individuals who were homozygous for the genotype (152). Smith et al reported that wild-type individuals had 5-methylTHF concentrations of 98-100%, with the proportion of non-methylTHF (consisting of 5, 10-methenylTHF, 5, 10-methyleneTHF, 5 and 10-formylTHF and THF) ranging from 0-2%. Individuals who were homozygous for the mutation, had proportions of non-methylTHF ranging from 8 to 72% (160). Bagley and Selhub (151) had similar results with wild-type individuals having proportions of 100% methylTHF and no formylTHF to total RBC folate and homozygous individuals showing proportions of 29 ± 22% of formylTHF to total RBC folate.

2.3.3.2.1. Reproduction

Currently, only two papers have examined circulating folate forms during reproduction. Obeid et al (161) reported serum folate form concentrations in non-pregnant controls and in 87 pregnant women at the time of delivery, 25 of whom took 400 µg FA during gestation and 61 who used no supplements at all. The group examined serum concentrations in both maternal and
umbilical cord serum using a stable-isotope dilution ultra performance liquid chromatography-mass spectrometry method to quantify form concentrations. They determined that supplemented pregnant women had the highest concentrations for total folate (P<0.041), 5-methylTHF (P<0.049) and formylTHF (P<0.001) but found no difference for THF or 5,10-methenylTHF when compared to both non-pregnant women and un-supplemented pregnant women (161). While the group did not calculate form distributions, it can be determined that the proportions of serum folate form concentrations relative to total serum folate in non-pregnant women were: 5-methylTHF (87.3%); formylTHF (0.8%); THF (11%); and 5, 10-methenylTHF (0.11%). In the non-supplemented pregnant women the proportions were: 5-methylTHF (85%); formylTHF (0.9%); THF (11.6%); and 5,10-methenylTHF (1.2%). In the supplemented pregnant women the proportions were: 5-methylTHF (92.8%); formylTHF (0.8%); THF (8.2%); and 5,10-methenylTHF (0.75%) (161). While it does appear that the non-supplemented pregnant women appear to have slightly higher concentrations of 5,10-methenylTHF, there is little variation in THF or formylTHF concentrations so no conclusions can be drawn. The second paper by Houghton et al (162) examined the effect of 5-methylTHF supplementation compared to folic acid supplementation and placebo during lactation (24). Women in this study consumed 1000 ug/d of folic acid throughout pregnancy. The distribution of major folate forms (THF, 5-methylTHF, 5-formylTHF, PteGlu) were measured by a stable-isotope dilution, liquid chromatography mass-spectrometry method (LC/MS) with electrospray ionization. Measurements were taken at 36 weeks gestation and after 16 weeks supplementation with 0 or 400 ug of folate (as 5-methylfolate or folic acid) from 1 to 16 weeks postpartum. In the placebo group (n=7), the distribution of folate forms consisted of 56-60% THF, 30-33% 5-methylTHF, 9-11% 5-formylTHF and <1% unmetabolized folic acid, results were similar for both supplemented groups (162). These results differed greatly from those reported for non-pregnant, non-lactating adults previously. The authors suggested a possible cause for this anomaly could have been due to physiological changes that occur in pregnancy, the high folic acid intakes of women in this study or the acidic pH used to prepare the samples caused form interconversions between the labile folate forms. The results of Study 1, presented herein, address the issues in Houghton et al (162)
2.4. FACTORS THAT INFLUENCE FOLATE CONCENTRATIONS

2.4.1. Folic Acid Supplement Use

2.4.1.1. General Population

It has been reported that in 2003-2006, 49% of the US population used some type of dietary supplements while 34.5% reported using a dietary supplement that contained FA (163, 164). Canadian data showed that in 2004, an estimated 40% used some type of nutritional dietary supplement and only 25% consumed a supplement that contained FA (59). Factors associated with likelihood of any type of dietary supplement use are: being female, being older, Caucasian background, food-secure household and a higher level of education (165).

2.4.1.2. Women and Reproduction

It is estimated that at any given time, approximately 27-32% of women of childbearing age are using FA supplements (55, 56). Based on data from NHANES 2003-2006, the median intake of folic acid in women aged 15-44 yr (from fortified foods and supplements) was 245 µg/d, with approximately 24% of women consuming the recommended amount of FA (55). In Canada, <5% of women of childbearing age who were consuming a FA supplement, in addition to dietary folate intake (both natural and fortified), had folate intakes below the recommendations (59). Evidence suggests that FA supplement use is the strongest predictor of ensuring RBC folate concentrations associated with reduction in NTD risk in women of childbearing years. Colapinto reported the odds of non-supplement users of having RBC concentrations ≥ 906 nmol/L was 53% (CI 0.24, 0.92) compared to supplement users among Canadian women of reproductive age, post-fortification of the food supply in Canada (54). The recommended dietary allowance of 400µg DFE FA/d for adults is based on the amount of folate required to maintain folate status, primarily assessed by RBC folate concentration. It has been shown that 400µg of supplemental FA, taken daily, can raise RBC folate concentrations above 906 nmol/L in as little as 8 weeks, and concentrations continue to increase the longer supplementation occurs (166, 167). However, lower daily doses of FA (140 µg/d) will also raise RBC folate concentrations above 906 nmol/L over time, so that by 40 weeks there is no
significant difference in RBC folate concentrations or in the odds of having RBC folate <906 nmol/L with intakes between 400 µg or 140 µg over the long term (167).

Despite the recommendations for women of childbearing age to use FA containing supplements, the actual number of women who regularly use supplements is less than one-third of women of childbearing years (55, 56). Evidence suggests that women are less likely to use supplements if they: are pregnant as teenagers; have education below high school level; have low income; smoke; are obese; are unemployed; less physical activity; have language barriers or were born outside of Canada (168, 169). Women who are more likely to take higher doses of FA supplements (> 1000 µg/d) are older (> 35 y), married or living with a partner, Caucasian, and have at least a college education (132).

While the average estimate for taking FA containing supplements in women of childbearing years at any given time is less than one-third, when American women are planning to become pregnant, the rate of FA supplement use increases to approximately 55% periconceptionally (132). During the first trimester, the rate of use is the same or higher than periconceptual use, with anywhere from 55-84% of women using FA supplements (40, 132). Supplementation is reported to be 76-78% during the second trimester and approximately 89% in the third trimester (40, 170). On average, 20-30% of women will not use FA containing supplements at all during pregnancy (132, 171). The evidence examining the factors that affect adherence to supplementation during pregnancy is limited, but supplement consumption is dependent on ethnicity and this interacts with several factors including gravidity, periconceptual supplement use, smoking and pregnancy-related nausea (172). Interestingly, another study reported that fear or experience of nausea, vomiting and gagging were the main reasons women reported limiting or discontinuing supplement use during pregnancy (173). While supplement use does impact on folate status, there is little evidence looking at what level of frequency of supplement use or the minimum dose of FA, taken on a regular basis, is required to improve women’s folate status to RBC folate concentrations associated with NTD protection.

2.4.2. Other Factors that Affect Folate Status

2.4.2.1. Diet

After supplement intake, diet is the major determinant of folate status in healthy women (174). The correlation coefficient for serum folate and dietary folate (as measured by semi
quantitative food frequency questionnaires) and serum concentrations is 0.61 and only 0.38-0.40 with RBC folate concentrations (175, 176). A more recent review of the literature found a wide variation in correlations reported in the literature (r=0.05-0.54), with the strongest correlations based on serum folate and taking supplement use into consideration (177). Typical folate intakes in countries without mandatory FA fortification are estimated to be approximately 250 µg DFE/d (74). A recent report of European dietary intakes in adults > 35 are estimated to be 250-350 µg/d for men and 200-300 µg/d for women. At the time this study was conducted not all European countries had mandatory fortification rules impacting total dietary folate intake (178). Data from the 2003-2006 NHANES reported usual folate intakes (mean ± SEM) of 772 ± 11 µg/d DFE from all folate sources including supplements, naturally occurring folates and fortified foods (60). The contribution from natural and fortified foods alone was 543 µg DFE/d (60). Canadian data from 2004 show that women of childbearing years between the ages of 19 –30y and 31- 50 y had estimated intakes of 407 ± 119 and 406 ± 137 µg DFE/d respectively, from naturally occurring and fortified-folate sources; if adjusted to reflect over-fortification practices, the intakes would be 475 and 471 µg DFE respectively (56). FA supplement users have a higher intake of dietary folate than those who do not use supplements across all ages (179). Further, diet quality has a significant effect on overall nutrient intake, those with more healthful dietary patterns generally having higher intakes of folate than diets higher in refined grains (even if fortified) and sweets and fats (397 vs 305 µg/d (180, 181). Higher diet quality, as determined by the consumption of dietary vitamins, minerals and trace elements, is associated with higher incomes and education, age, increased food diversity and physical activity (180).

2.4.2.2. Genotype

Habitual dietary or supplemental intake of a nutrient are prime determinants of nutrients status in healthy individuals; however, it is being increasingly recognized that other factors, such as genetics can have a significant effect on overall status (174). The most common single nucleotide polymorphism associated with impaired folate status is the C677T polymorphism in the MTHFR gene. MTHFR is the enzyme that converts 5, 10-methyleneTHF to 5-methylTHF. The 677T allele produces a thermolabile enzyme with a 50-60% reduction in enzymatic activity reducing the amount of 5-methylTHF generated to remethylate homocysteine to produce methionine (104, 105). Approximately 10-15 % of the North
American Caucasian population are homozygous for this polymorphism (151). Individuals who are homozygous for the C677T variant have higher folate requirements in order to compensate for the reduced MTHFR activity. Thus, at inadequate or marginal folate intakes, homocysteine can be elevated in those who are homozygous for the C677T variant. Studies have provided inconsistent evidence of an increased risk for cardiovascular disease in adults with the 677C→T polymorphism in conjunction with these elevated homocysteine levels (1). Further, the C677T MTHFR polymorphism is associated with an increased risk for NTD, Down’s syndrome, certain complications in pregnancy (3), a possible link with autism spectrum disorders (137, 182, 183), and even schizophrenia (184) in conjunction with lower folate concentrations or altered folate metabolism. Other variants in genetic polymorphisms (such as MTHFR 1298 A-C and RFC1 80A-G variants) are less well studied and do not appear to affect serum or RBC folate status (185).

2.4.2.2.1. NTD Risk

Individuals who are homozygous for the C677T MTHFR genotype have an increased risk of NTD-affected births, although the evidence is somewhat conflicting and may be confounded by factors including race and gene-nutrient interactions (3). It has been estimated that women who are homozygous for the variant have a 60% increased risk of having an infant with NTD while homozygous offspring have a 90% increased risk of being born with an NTD (105). Even women who are heterozygous for the variant have a slight increase in risk (10%) while heterozygous infants have a 30% increase in risk (105). While there is an increased level of risk in individuals who are homozygous for the 677 C>T variant, the risk is highest only if folate concentrations are in the low-to-normal range along with corresponding elevated homocysteine concentrations.

Those who have a diminished response to dietary/supplement intakes may require larger doses to gain an equivalent level of protection from folate-affected NTD (186). This is one of the reasons that Health Canada suggests that women with a pregnancy affected by NTD or a family history with NTD may require a higher dose of FA (97).

2.4.2.3. Other Sociodemographic and Lifestyle Factors that can Influence Folate Status
While supplement use, dietary choices, and genetics are the most significant factors that can impact on folate status, there are other factors that have been shown to either positively or negatively influence nutrient status. These can include: ethnicity, smoking and alcohol use, education and income, and medications. Research from the US shows differences in RBC folate concentrations by ethnic background. There is a significant difference in RBC folate concentrations between Mexican-American, non-Hispanic Black and non-Hispanic White ethnicities in the 2003-2006 NHANES with non-Hispanic Blacks having the lowest concentrations (487 nmol/L) while non-Hispanic Whites having the highest concentrations at 650 nmol/L (187). There is some question as to whether these differences are from dietary choices, not using FA containing supplements, or if there is a genetic component. Some ethnic populations have higher rates of the MTHFR C677T variant than the average 10-15% of the general population, putting these populations at increased risk of low folate status even with an adequate intake of dietary folate. In a controlled feeding study, men of Mexican-American background were given 438 µg/d DFE of folate and varying doses of choline at or above the AI (188). At the end of the study, of the T/T group, 34% were folate deficient (< 6.8 nmol/L) and 79% had elevated homocysteine concentrations (> 14 µmol/L) compared to 16% deficient and 7% elevated in the C/C group (188). While African American women were found to have significantly (p≤0.05) lower blood folate concentrations and excrete less urinary folate in a folate depletion/repletion study than women of Mexican-American or non-Hispanic White backgrounds, all were wild-type variant for C677T MTHFR (189).

Evidence suggests that low serum folate is associated with factors consistent with an unhealthy lifestyle: smoking, alcohol intake (both low and high), high coffee intake, and an unhealthy diet (190). Smoking has been shown to have an adverse effect on both serum and RBC folate concentrations (Spearman correlation coefficient; -0.31 and -0.29, P<0.05) respectively; however, alcohol consumption was only significant with serum folate (-0.08, P<0.05) (187). Daily smokers have 1.54 times the odds of being folate deficient than those who do not smoke (190). As well as having lower folate concentrations than non-smokers, smokers may also have significantly altered folate form distributions (higher proportions of formylTHF and 5, 10-methenylTHF and lower proportions of THF and 5-methylTHF) and higher concentrations of folic acid than non-smokers (191). Smoking during pregnancy may also negatively influence folate transport to the fetus (192). A negative correlation (r = -0.31,
P=0.019) between cigarettes smoked and 5-methylTHF concentrations in venous cord blood, and 5-methylTHF venous cord concentrations were significantly lower in smoking mothers than in non-smoking mothers (15.1± 7.6 ng/ml vs 19.0 ± 7.0 ng/ml, P=0.0498) (192).

Alcohol has been demonstrated to have complex relationship. The detrimental effects of chronic high alcohol intake on folate metabolism are well established. The main adverse effects are: decreased expression of intestinal reduced folate carriers and passive transport in the jejunal brush border membranes leading to decreased intestinal absorption of folates; alterations in hepatic basolateral membranes affecting folate metabolism and storage; increased urinary folate excretion in response to both acute and chronic alcohol intake; and, lower folate concentrations (193). There is some evidence suggesting that high alcohol intake may have some epigenetic effects by inducting aberrant DNA methylation which could decrease SAM and increase homocysteine concentrations and induce alcohol-related carcinogenesis potentially through increased oxidative stress or low dietary intake of one carbon nutrients, although the mechanism is not well understood (194). While frank folate deficiency in alcoholics is no longer the norm in countries that have mandatory folate fortification, mean folate concentrations remain low (193). An inverse linear pattern is seen between having 2 or more drinks per day (~ 15 g alcohol/drink) and RBC folate concentrations, with significantly lower concentrations than those who do not drink (256 [95% CI 243, 269] vs 280 [95% CI 272, 288] µg/L (195), P=0.0111) respectively (187). However, some studies demonstrate a J-shape effect between alcohol and risk, with moderate intakes of alcohol having a protective effect on homocysteine and some diseases such as coronary artery disease; further, high intakes of folate enhance the protective effect against the deleterious effects of high alcohol intakes (190, 196-198). Part of this protective effect may be due to the presence of B vitamins in beer which contributes to total folate intake and can reduce homocysteine concentrations (199). Data collected on food sources to nutrient contribution found that alcohol/beer contributes approximately 0.9-2.4% of total folate intake and 6% of vitamin B6 intake (62, 88, 200). Data from Danish adults found an inverse association between risk of low folate concentrations and alcohol intake, but when stratified by type of alcohol, the protective effect is found only with beer and not wine (190). Currently, there is little evidence looking at alcohol intake in conjunction with a comprehensive list of other factors demonstrated to influence folate status in women of childbearing years to determine what factors have the most influence on folate status.
Education has been shown to have a positive effect on folate status with those with > high school having significantly higher RBC folate concentrations than those with < high school (279 vs 252 µg/L [632 vs 571 nmol/L](187). Higher incomes are also correlated with higher folate status (Spearman r=0.14, P<0.05)(187). However, it should be pointed out that both increased supplement use and higher dietary quality are also associated with higher education and income.

Certain medications are known to have an adverse effect on folate metabolism. These include the cancer drug methotrexate, as folic acid may interfere with its anticancer effects; anticonvulsants, which may reduce serum folate concentrations; some antibiotics; and some medications used in the treatment of ulcerative colitis, which can inhibit the absorption of folate (42, 201). Use of these drugs requires additional supplementation in the form of either FA or 5-methylTHF.

2.5. RISKS ASSOCIATED WITH EXCESS INTAKE OF FOLIC ACID

2.5.1. Masking B_{12} Deficiency and Exacerbating Effects of Low B_{12} Status

The benefits of FA have been well documented; however, there is a growing body of evidence suggesting that consistently high intakes may have a number of adverse effects in the adult population. One of the main adverse effects associated with high folic acid intakes is that it can mask vitamin B_{12} deficiency. Both nutrients are metabolically interconnected where B_{12} is required by methionine synthase to regenerate methionine from homocysteine with the methyl group coming from 5-methylTHF (see Figure 2.2). As the conversion of 5,10-methyleneTHF via MTHFR is irreversible, if there is an insufficient supply of B_{12}, the activity of methionine synthase is reduced, the concentrations of SAM are diminished, folate is trapped and cannot be used for DNA synthesis or cell division (45). The result is hematological changes resulting in megaloblastic anemia; the identical physiological response to folate deficiency (42, 45). Even if an individual is supplemented with FA, homocysteine can’t be converted to methionine (methyl trap) however FA can be converted to THF which can then be utilized for purine synthesis and subsequently DNA biosynthesis. As DNA biosynthesis proceeds, the characteristic megaloblastic anemia associated with vitamin B_{12} deficiency does not occur. The absence of
megaloblastic anemia delays the diagnosis of vitamin B\textsubscript{12} deficiency. In the absence of remethylation of homocysteine to methionine there is a reduction in SAM. One of the roles of SAM is methylation of myelin (202). Vitamin B\textsubscript{12} is also involved in the conversion of methylmalonyl-CoA to succinyl-CoA. Failure of this conversion from B\textsubscript{12} deficiency results in an increase of methylmalonic acid, a myelin destabilizer (203). Demyelination of neural tissue will continue as long as the B\textsubscript{12} deficiency is not corrected and the neurological damage could be permanent (202).

As well as masking B\textsubscript{12} deficiency, there is a growing body of evidence suggesting that high folate intake can aggravate the side effects of sub-optimal B\textsubscript{12} status including cognitive or neurological impairment. Groups of concern for potential cognitive issues are those with low B\textsubscript{12} intake, those who experience some degree of malabsorption of vitamin B\textsubscript{12}, and older adults, particularly those who are elderly, as they are more vulnerable to both age-related declines in B\textsubscript{12} absorption and increased risk of dementia, a common neurodegenerative disease associated with aging (204). There are a number of studies that are cross-sectional, longitudinal and intervention in design which link low B\textsubscript{12}, low folate and/or high homocysteine to increased dementia and/or cognitive impairment; the evidence is somewhat variable and causality has not been conclusively proven (20). There is even some longitudinal evidence suggesting that with long-term hyperhomocysteinemia, dietary folate (not supplements) may actually have a positive protective effect on cognition (205). However, there is also a body of evidence suggesting that high serum folate in conjunction with low B\textsubscript{12} status can exacerbate cognitive dysfunction. Evidence from 1999-2002 NHANES found that having a low B\textsubscript{12} status (< 148 pmol/L) does increase the risk of cognitive impairment (OR=2.5, 95% CI: 1.6, 3.8) but when combined with a high serum folate (> 59 nmol/L), the odds of impairment are increased further (OR=2.6, 95% CI: 1.1, 6.1, P<0.001) (206). The evidence is otherwise in those with normal B\textsubscript{12} status (> 148 pmol/L) where normal to high serum folate concentrations > 59 nmol/L were found to actually be protective (OR=0.5, 95% CI: 0.2, 0.96, P=0.005) of cognition (206). A more recent study by the same group using data from the Framingham Heart Study found a more rapid rate of cognitive decline in those whose plasma B\textsubscript{12} was < 258 pmol/L and plasma folate concentrations > 20.2 nmol/L (P<0.001)(207). They further found a similar phenomenon over an 8 year period with FA supplement use and low B\textsubscript{12} status (P=0.002) (207). A recent Australian paper combined the results of 3 cohort studies and found that those with low B\textsubscript{12} status (defined as <
250 nmol/L) and either normal (≤ 1594 nmol/L) or high (> 1594 nmol/L) RBC folate had an increased risk of cognitive impairment (AOR=1.85, 95% CI: 1.37-2.50; P<0.001) and (AOR=3.45, 95% CI: 1.60-7.43; P=0.002) respectively (21). While the exact mechanism is unknown, the evidence suggests that there is a further increase in cognitive impairment in individuals with low or sub-optimal B\textsubscript{12} concentrations and a high folate intake from either dietary or supplemental sources compared to the cognitive decline associated with low B\textsubscript{12} status alone.

2.5.2. Unmetabolized Folic Acid, Cancer, and Other Issues

While masking B\textsubscript{12} deficiency from high intakes of FA is the most often cited area of concern, there is a growing body of literature suggesting potential harm in other areas including unmetabolized FA, certain cancers, immune function and physiological adaptations. There have been a number of studies discussing the presence of FA in circulation. FA is a synthetic form of folate used in supplements and fortified foods because of its structural stability in contrast to the labile nature of naturally occurring folates. FA must be reduced and methylated to THF in order to be utilized metabolically. The concern is that any potential adverse effects are currently unknown. One of the earliest studies detecting the presence of unmetabolized FA in serum was by Kelly et al (82) who found unmetabolized FA in subjects with a threshold intake of 266 µg from fortified breakfast cereals. Later, it was determined that at an average intake of < 200 µg/d is unlikely to result in the consistent presence of FA in circulation while doses of 400µg/d result in the presence of unmetabolized FA for at least 6 hours in part due to the extremely low conversion rate of FA to DHF then to THF in humans (18, 80). In 2001-2002, an estimated 38% of the American population ≥ 60 yrs had detectable concentrations of FA with a mean concentration of 4.4 ± 0.6 nmol/L; this included a significant number of supplement users (~60%) (19). The current controversy associated with the presence of the synthetic form in circulation is over whether or not there are any adverse health effects.

Folate has been shown to have a dual role in cancer development. Epidemiologic evidence suggests that high dietary folate intake can be protective against certain cancers such as lung, colorectal and breast cancer (22, 23). In folate deficiency, DNA synthesis and methylation are impaired which can increase structure instability, mutations, strand breakage and altered
methylation in DNA all of which are linked to increased risk of cancer development (195). Improving dietary intake of folate and FA supplements would be protective against these damaging effects. However, depriving cancer cells of folate to synthesize DNA is also an established treatment for cancer; animal studies suggest that the suppression or promotion of tumour growth from folate may depend on the tissue, the presence of cancerous cells and dose of folate given (23, 24, 201). It is thought that high intake of supplemental folate could potentially provide an increased pool of nucleotide substrate thereby promoting folate metabolism and promoting proliferation especially in precancerous cells (23). Currently the evidence is somewhat contradictory. After mandatory fortification of FA in both Canada and the United States in 1998 there was significant increase in cases of colorectal cancer that is not entirely explained by improved diagnostic methods but has been attributed to the increase in FA in fortified foods (208). Further, a bell-shaped relationship between plasma folate concentrations and colorectal cancer risk was demonstrated in the Northern Sweden Health and Disease Cohort with those in the highest quintile of serum folate (> 11.3 nmol/L) having 3.87 times the risk in the odds of developing colorectal cancer compared to those in the lowest category (< 5.1 nmol/L) (209). More recently, an Australian study showed that high dietary folate from naturally occurring sources was protective against colorectal cancer in those with low methionine intake (HR=0.63) but an increased risk (HR= 1.36) when intakes of both nutrients were high suggesting that other nutrients in one-carbon metabolism may also play a role in cancer development (210). Two recent meta-analysis papers looked at FA and risk of cancer. The first meta-analysis (211) looked at the outcome of placebo-controlled trials of FA (doses ranged between 0.5 to 5 mg) for prevention of heart disease (10 trials) or colorectal adenoma (3 trials) over an average of 5.5 years. There was no statistically significant effect on cancer development at any specific site (risk ratio=1.06; 95% CI 0.99-1.13) for lung, breast, prostate or colon for a weighted mean follow-up of 5.5 years (211). In the second analysis (212) FA supplementation ≥ 400 µg/d and cancer incidence and/or cancer mortality were examined (19 studies- 12 randomized control trials). They found a borderline significance of total cancer incidence (RR=1.07; 95% CI 1.00 to 1.14) with prostate cancer being the only site-specific cancer to show an increase in risk (RR=1.24; 95% CI 1.03 to 1.49) with FA use (212). There is some evidence that genetic susceptibility can also impact risk of cancer development with varying folate intakes. A 2004 paper by Sohn et al. first demonstrated that genetic polymorphisms can have functional
consequences with altered chemosensitivity to colon and breast cancer cells for the homozygous C677T MTHFR cells compared to wild type (213). A more recent meta-analysis found that the A1298C polymorphism had no influence on colorectal cancer risk, but those who were homozygous for the C677T MTHFR genotype had a reduced risk of colorectal cancer with higher intakes of folate intake (0.63, 95% CI: 0.41-0.97) (214). In another study, a low folate intake in conjunction with high genetic risk (C677T, A1298C, and A2756G) was found to increase the odds of cervical neoplasia (OR=1.67, 95% CI: 0.92, 3.04, P-trend <0.001)(215).

At this time, the role of folate in cancer and cognitive impairment are the most researched of potential adverse effects associated with long term high folate intake in the adult population. There are a few studies that have examined other potential health risks from repeated exposure to high folate. In post-menopausal women, an inverse relationship was seen between higher serum folate concentrations (≥ 3 nmol/L) and natural killer cell cytotoxicity with a significant reduction of approximately 23% (P=0.04) (25). An index of immune function, natural killer cells are responsible for fighting viral infections and cancer cells. This inverse relationship was more pronounced in older women with high concentrations with reductions up to 25% (25). No effect was seen for either serum total folate or 5-methylTHF (25). A more recent study examining supraphysiologic serum folate concentrations in both adult men and women in Chile found no association between serum folate or B12 and NK cell activity (26). Supraphysiologic concentrations for serum folate are above 45 nmol/L (201). Work with cell lines demonstrated a significant down-regulation in both intestinal and renal uptake of folate when exposed to high amounts of folic acid over time (216).

2.6. OTHER NUTRIENTS INVOLVED IN ONE-CARBON METABOLISM

2.6.1. Description and Metabolic Functions

While the main focus of this literature review has been on the vitamin folate, there are other nutrients inextricably linked to one carbon metabolism. These nutrients include: vitamins B2 (riboflavin), B6 and B12; choline and betaine; and methionine (see Figure 2.2). Methionine, choline, folate and B12 are involved in the remethylation of homocysteine for the production of methionine and subsequently SAM; while B6 is involved in the conversion of THF to 5, 10-methyleneTHF and the transsulfuration pathway (liver only); and B2 is cofactor for MTHFR
(174). Derangements to 1-carbon metabolism at different life stages are thought to be associated with a number of different adverse outcomes including cancer, non-alcoholic fatty liver disease, cognitive decline and so on (102). Inadequate intakes of folate, B₆, B₁₂, and choline can disrupt one carbon metabolism thereby raising homocysteine concentrations and reducing methylation, both of which are potential mechanisms for NTD (174). Disruptions highlight the interrelationship between these nutrients as compensatory changes occur to enzymes and other nutrients in the pathways (217). Vitamin B₁₂ deficiency can create a pseudo folate deficiency via the “methyl trap” as described earlier. Folate will be trapped in the 5-methylTHF form (see Figure 2.2) and cannot be used for methylation or DNA synthesis (45). Folate deficiency can cause secondary hepatic choline deficiency (217) while hepatic folate content has been known to decrease 30-40% in rats on a choline deficient diet (42). Patients on total parenteral nutrition require the addition of choline, otherwise they will develop fatty liver and liver damage, even if they consume adequate amounts of folate, methionine and B₁₂ (42, 217). Pregnant women with low vitamin B₁₂ concentrations were found to have lower concentrations of plasma free choline and betaine at 36 weeks of gestation compared to those with normal concentrations in a folate replete population (218).

2.6.1.1. Vitamin B₆

Vitamin B₆ is the term used to describe 7 related compounds: pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) and their respective 5’ phosphates (ie. pyridoxal 5’phosphate [PLP], pyridoxamine 5’ phosphate [PMP]), and pyridoxic acid (as 4-pyridoxic acid in urine) (42, 219). The most prominent forms found in humans are PLP and PMP, the majority of which is found in muscle bound to phosphorylase, whereas in plants the prominent forms are PN and its 5’ form (42, 219). In the body, PLP is the metabolically-active form and is involved in over 100 enzymatic reactions; it has an important role in brain neurotransmitter metabolism, particularly in the developing brain; catalyzes the first step in heme synthesis, and acts as a coenzyme in all transamination reactions and in decarboxylation and deamination of amino acids (95, 220). PLP also modulates the transcription responses of various sex hormones and glucocorticoids (219).

Metabolically, pyridoxine is involved in both one carbon metabolism (via the conversion of THF to/from 5, 10-methyleneTHF) and the transsulfuration pathway which breaks down
homocysteine (see Figure 2.2). PLP is linked to riboflavin, B<sub>12</sub> and folate; positive correlations have been shown in cerebrospinal fluid between PLP and 5-methylTHF (219).

Vitamin B<sub>6</sub> deficiencies are generally considered to be rare; however a recent NHANES report examining nutrient biochemical indicators found that the prevalence estimates for risk of B<sub>6</sub> deficiency was ~11% for the population but the rate was somewhat higher in individuals > 40 y and in women (221). Clinical symptoms of deficiency include seborrheic dermatitis, microcytic anemia and convulsions (42, 220). It should be noted however, that B<sub>6</sub> status is determined via serum PLP concentrations, and the cut-off concentration (20 nmol/L) is not accompanied by any overt symptoms of deficiency (42). As mentioned, the major form of B<sub>6</sub> in circulation is PLP and this is the form most often used as an indicator of B<sub>6</sub> status, as there is currently no rapid or simple method to determine all forms of B<sub>6</sub> (174, 219). The role of vitamin B<sub>6</sub> in amino acid metabolism makes adequate intake during pregnancy crucial. During pregnancy, the fetus is dependent on maternal supplies of B<sub>6</sub> through the placenta; concentrations of plasma PLP decline almost threefold compared to non-pregnancy concentrations, primarily in the third trimester (42, 219). To prevent the decline in maternal PLP concentrations requires approximately 2 mg/d of supplemental PN in the first trimester and up to 10 mg/d during the last trimester (92). The placenta transfers pyridoxal in both directions; however, evidence suggests that the transfer is much higher towards the fetus and umbilical vein concentrations have been found to be approximately 9 times that of maternal concentrations (219). Evidence suggests that this high demand for vitamin B<sub>6</sub> is based on the fetal demand for amino acids which far exceeds the amount necessary for growth; the overage is thought to be utilized in energy metabolism and synthesis of other molecules (219).

2.6.1.2. Vitamin B<sub>12</sub>

A water-soluble B vitamin, B<sub>12</sub> or cobalamin is the term used to describe a related group of compounds (corrinoids) containing a central cobalt atom surrounded by a heme-like corrin ring structure and attached to a phosphoribo-5,6-dimethylbenzimidazolyl base (42, 92). There are two metabolically-active cobalamin coenzymes in the body: methylcobalamin and 5-deoxyadenosylcobalamin, each used in two key metabolic reactions in the body (42). The methylation pathway uses methylcobalamin for the enzyme methionine synthase (see Figure
2.2) while the energy metabolism of odd chain fatty acids and proteins and hemoglobin synthesis make use of adenosylcobalamin in L-methylmalonyl-CoA mutase to convert L-methylmalonyl-CoA to succinyl Co-A (succinyl Co-A is a precursor for heme) (42, 222).

Unlike the other nutrients discussed in this literature review, cobalamin has a somewhat complicated absorption process requiring: 1) release from protein matrix via stomach acid and pepsin; 2) immediately binding to haptocorrins called R proteins to protect it from denaturing; 3) then is released in the more neutral pH of the duodenum; and, 4) immediate binding to intrinsic factor (a glycoprotein produced in the stomach by parietal cells) to be absorbed in the terminal ileum(202). It then enters circulation where approximately 50% is stored in the liver (202). Normal liver stores of vitamin B$_{12}$ can last from 3 to 5 years in replete subjects (202). Cobalamin is absorbed by both the active intrinsic factored mediated system and by passive absorption. Total absorption decreases as vitamin B$_{12}$ intake increases. The active intrinsic factor-mediated system is estimated to absorb all free vitamin B$_{12}$ at intakes between 1.5- 2.0 mg; at intakes above this level passive diffusion will account for ~1-3% being absorbed of the remainder of the dose (42).

The major symptoms associated with vitamin B$_{12}$ deficiency are neurological abnormalities (peripheral neuropathy, cognitive disturbances, ataxia and sensory disturbances) and macrocytic anemia develops from dysfunction in one carbon metabolism. Macrocytic anemia from B$_{12}$ deficiency is clinically-identical to that caused by folate deficiency with impaired DNA synthesis affecting RBC formation (42, 92). Methylation of myelin proteins is an important outcome from adequate functioning of the methylation pathway and the impact of prolonged B$_{12}$ deficiency is demyelination (92). B$_{12}$ deficiency will also impact the enzyme methyl malonic-CoA mutase leading to elevated concentrations of methylmalonic acid and malonyl-CoA which inhibits β-oxidation affecting both mitochondrial energy and lipid metabolic metabolism (223).

Like the other one carbon nutrients, requirements for vitamin B$_{12}$ increase during pregnancy to meet the demand for growth, development and physiological functions through synthesis and methylation. Serum B$_{12}$ concentrations decline during the first trimester, more than can be accounted for by hemodilution, and can potentially promote a state of deficiency (42, 92, 224). The rate of transmethylation is higher in the latter part of pregnancy increasing the demand for all methyl donors (223). This is supported by further decreases in vitamin B$_{12}$
concentrations (42). Fetal requirements for vitamin B₁₂ are estimated to be 0.1-0.2 µg/d and during pregnancy, the fetus absorbs B₁₂ through the placenta (92, 222). The placenta concentrates B₁₂ which is then transferred to the fetus (42). Low maternal B₁₂ concentrations decrease methylation and this is linked with some of the adverse effects on fetal development such as NTD (see below), long-term cognition and poor growth outcomes. Evidence suggests that long term dietary restrictions of animal products has an adverse effect by decreasing serum B₁₂ and increasing homocysteine concentrations which are improved significantly with increasing B₁₂ intake to a cut off of 3 µg/d (225).

2.6.1.3. Choline

Both choline and folate are the major methyl donors in the methylation cycle in one carbon metabolism. Choline is an essential nutrient important for the structural integrity of cell membranes, neurotransmitter synthesis (acetylcholine), lipid and cholesterol transport and metabolism (lipoproteins), transmembrane signalling (phospholipids), as well as a methyl donor (42, 217). The role of choline in lipid transport is the reason that choline deficiencies lead to excessive accumulation of triglycerides in the liver even when there is sufficient supply of the other one carbon nutrients (217). Dietary choline can be acetylated to form acetylcholine, phosphorylated to form phosphatidylcholine or can participate as a methyl donor to form betaine. The majority of choline is converted to phosphatidylcholine and is the predominant phospholipid (>50%) in mammalian cell membranes (217). Choline is oxidized in the mitochondria to become betaine aldehyde and then betaine in an irreversible reaction (220). It is betaine that can be used to remethylate homocysteine leading to SAM formation and thereby reducing homocysteine concentrations (217, 220). Average fasting plasma choline concentrations are approximately 10 µmol/L with a range of 7-20 µmol/L (42). Plasma choline will vary in response to dietary intake and can increase 2-4 fold in response to high dietary or supplemental choline intake (42). At low plasma free choline concentrations, choline is incorporated into phospholipids but at higher concentrations most choline will be converted to betaine within the liver (226).

In reproduction, choline requirements increase; phosphatidylcholine is necessary for cell division and growth, sphingomyelin is necessary for the myelination of nerve fibers,
acetylcholine influences neurotransmitter synthesis and transmembrane signalling and may modulate cellular proliferation and differentiation, as well as increased methylation of DNA, proteins and so on (226, 227). In early pregnancy, both the fetus and placenta lack the enzyme for de novo choline synthesis therefore adequate maternal supply is critical for fetal development. In the latter part of gestation, fetal concentrations of choline have been found to be approximately three times higher than maternal concentrations to enhance the uptake of choline by the developing brain, central nervous system and other tissues (226, 227). A maternal diet high in choline in the early stages of gestation was found to reduce the risk of NTD (see below) and to enhance visuospatial and auditory memory in rats and these changes were lifelong (228). More recently, maternal choline concentrations at 16 weeks gestation were positively associated with infant cognitive test scores on the Bayley Scales of Infant Development at 18 months of age while maternal folate and B₁₂ status were not related (229), while another study found that maternal intakes of ~80% of the AI for choline plus a phosphatidylcholine supplement of 750 mg/d after 18 weeks of gestation and for the duration of pregnancy were not associated with enhanced infant brain function at 12 months of age (230). Post-gestation, serum choline concentrations are approximately 50-100% higher in breastfeeding women in order to accommodate choline uptake into the mammary glands for milk production (42, 226).

2.6.2. Food Sources, Dietary Intake and Blood Values

2.6.2.1. Vitamin B₆

Vitamin B₆ is found in both plant and animal foods. In animal foods it is primarily found as pyridoxal phosphate associated with glycogen phosphorylase in muscle, while in plants it is most often seen as pyridoxine and pyridoxine phosphate (219). It is widely distributed in foods including muscle meats, breakfast cereals (through fortification), vegetables, and fruits (219). Rich sources can include poultry, legumes, nuts and whole grains (220). Evidence suggests that the bioavailability of B₆ in a mixed diet is approximately 75% while the absorption from supplemental B₆ is comparable (219). B₆ is not stored in the body for any length of time, so regular intake to maintain status is necessary (42).
Requirements for B₆ are based on maintaining a plasma concentration for 5’-pyridoxal phosphate (PLP) of at least 20 nmol/L and assuming a bioavailability of 75% in a mixed diet in adults (42). This is the vitamin form found most often in tissues, is reflective of liver stores, corresponds well to other markers of vitamin B₆ status, and is relatively slow (approximately 10 days) to respond to changes in nutrient intakes (42). Factors that may affect requirements for vitamin B₆ include: some drugs used to treat tuberculosis, tetracycline, antidepressants and some chemotherapy medications (42). Requirements for women of childbearing years and pregnancy are shown in Table 2.3, as are population dietary intakes for both Canada and the United States and calculated prevalences of inadequate intakes (when available). In general, dietary intakes of vitamin B₆ are above requirements for most women of childbearing age in both countries, with approximately 13% having intakes below the EAR (59, 60).

Table 2.3 Dietary Recommended Intakes, estimated dietary intakes (food sources only) and percentage not meeting recommendations for B₆, B₁₂ and choline in women of reproductive age

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>EAR/AI²</th>
<th>Data Source</th>
<th>Year of Survey</th>
<th>Mean Dietary Intake of Women (&gt;19 yrs)</th>
<th>Prevalence of Inadequate Intake (% ± SE)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₆</td>
<td>1.1 mg/d 1.6 mg/d</td>
<td>CCHS 2.2</td>
<td>2004</td>
<td>1.59-1.65 mg/d#</td>
<td>13 ± 2</td>
<td>(59, 231)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHANES</td>
<td>2003-2006</td>
<td>1.6 mg/d#</td>
<td>27 ± 3</td>
<td>(60, 179)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHANES</td>
<td>2009-2010</td>
<td>1.8 mg/d #</td>
<td>n/a</td>
<td>(232)</td>
</tr>
<tr>
<td>B₁₂</td>
<td>2 µg/d 2.2 µg/d</td>
<td>CCHS 2.2</td>
<td>2004</td>
<td>3.4-3.9 µg/d</td>
<td>12 ± 3</td>
<td>(59, 231)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHANES</td>
<td>2003-2006</td>
<td>4.1 µg/d*</td>
<td>8 ± 1.4</td>
<td>(60, 179)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHANES</td>
<td>2009-2010</td>
<td>4.7 µg/d*</td>
<td>n/a</td>
<td>(232)</td>
</tr>
<tr>
<td>Choline</td>
<td>425 mg/d 450 mg/d</td>
<td>NHANES</td>
<td>2007-2008</td>
<td>260 mg/d</td>
<td>n/a</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHANES</td>
<td>2009-2010</td>
<td>269-277 mg/d</td>
<td>n/a</td>
<td>(232)</td>
</tr>
</tbody>
</table>

¹ CCHS 2.2, Canadian Community Health Survey 2.2; NHANES, National Health & Nutrition Examination Survey
² Both B₁₂ and B₆ have an Estimated Average Requirement (EAR) while choline has an Adequate Intake (AI)
# Includes both naturally occurring and fortified food sources.
* In the United States, in addition to naturally occurring sources, most ready-to-eat cereals are fortified with B₁₂ at either 25% DV (1.5 µg) or 100% DV (6 µg)(233).

The accepted biomarker of vitamin B₆ nutrition status is PLP. There are limited data examining PLP concentrations on a population level. Using NHANES 2003-2006, two studies (the first time PLP was measured) looked at PLP concentrations in the American population. In women aged 21 to 44 yr who have never used hormones (e.g. HRT or oral contraceptives) the
mean (range) PLP concentration was 30 (26-35) nmol/L with significant differences in PLP concentrations between the highest (>5 mg/d) and lowest (<2 mg/d) level of vitamin B₆ intakes (234). More recently, it was reported that the prevalence of B₆ deficiency, using serum PLP, was 11% for the U.S. population; females had a slightly higher rate of deficiency compared to males (>10%) and older adults were more likely to be deficient than young adults (16% vs 9.9%) (221). However, it should be pointed out that, while PLP concentrations may suggest that 1/10 of the population is B₆ deficient, overt clinical symptoms of B₆ deficiency are rare, suggesting that while PLP may be a sensitive measure of systemic B₆ status, overall low concentrations may not necessarily reflect deficiency (92, 174).

At this time there is little up-to-date information that describes the dietary sources in women of childbearing years and no information looking at food sources in Canadian women. Recent dietary trends have made high intakes of red meat less desirable and the differences in fortification regulations for breakfast cereals in Canada would mean that these products would have a much smaller contribution to the B₆ dietary intake of Canadian women.

Table 2.4 summarizes data from the 1976-1980 NHANES (62) which describes the top 5 dietary sources of B₆ for adults aged 19 to 74.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Description</th>
<th>% total B₆</th>
<th>Cumulative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beef steaks &amp; roasts</td>
<td>9.1</td>
<td>9.1</td>
</tr>
<tr>
<td>2</td>
<td>Alcoholic beverages</td>
<td>6.0</td>
<td>15.1</td>
</tr>
<tr>
<td>3</td>
<td>Potatoes (not fried)</td>
<td>6.0</td>
<td>21.1</td>
</tr>
<tr>
<td>4</td>
<td>Breakfast cereals (fortified)</td>
<td>5.0</td>
<td>26.1</td>
</tr>
<tr>
<td>5</td>
<td>Pork chops &amp; roasts</td>
<td>4.1</td>
<td>30.2</td>
</tr>
</tbody>
</table>

¹ Data derived from (62).

2.6.2.2. Vitamin B₁₂

All vitamin B₁₂ is synthesized by bacteria and algae. Humans acquire it when fermentation in the gastrointestinal tract of herbivores produces a wide variety of
microorganisms. These microorganisms then synthesize various forms of B₁₂ which are then absorbed by the animal and incorporated into their tissue (202). Thus the main food sources for cobalamin are animal foods (meat, poultry, fish, dairy and eggs) and select fortified foods such as plant-based beverages (e.g. soy milk) and simulated meat and poultry products. In the United States, the fortification of ready-to-eat breakfast cereals is also permitted. Fortified foods and supplements use cyanocobalamin which is metabolized to the active forms in cells with an estimated bioavailability of 60% in doses < 5 µg (42, 202). Estimated bioavailability for B₁₂ in naturally-occurring in foods is approximately 50% with bioavailability ranging from < 9 % for eggs up to 89% for mutton (235). While edible algae (laver, chlorella and spirulina) contain large amounts of B₁₂, most is pseudovitamin B₁₂ and is metabolically inactive (235).

The requirements for B₁₂ intake are based on the amount needed in order to maintain normal hematological status (defined as being a stable hemoglobin concentration and a normal mean cell volume) and B₁₂ serum concentrations ≥ 150 pmol/L (suggested as the cut-off which is 2 SD below mean and is associated with clinical manifestations of deficiency) (174). Other biomarkers associated with low B₁₂ status include homocysteine and methylmalonic acid concentrations; however homocysteine is not specific for B₁₂ and methylmalonic acid, while specific, is not really feasible, as it requires specialized equipment and is costly to perform (236). Factors that may affect requirements include: age, low dietary intake, prevalence of atrophic gastritis, medications that affect stomach acidity, pernicious anemia (affects about 1% of the population), malabsorption, smoking and gender (males have lower concentrations then females) (42). Requirements for women of childbearing years and pregnancy are shown in Table 2.3 as are population dietary intakes for both Canada and the United States and calculated prevalences of inadequate intakes (when available). In general, dietary intakes for vitamin B₁₂ are above requirements for women of childbearing years. The prevalence of inadequate intake is higher in Canada than in the United States, due to the fact that the US allows fortification of ready-to-serve breakfast cereals. It has been estimated that foods fortified with B₁₂ provide an additional 1 µg per day in additional to naturally occurring sources (60).

Serum vitamin B₁₂ concentrations in the US population have been reported in the NHANES surveys for over a decade. In 1999-2002, the prevalence of deficiency (< 148 pmmol/L) was ≤ 3 % for those 20-39 y, increasing to 6% in those ≥ 70 yrs. At the same time marginal depletion (148-221 pmol/L) was more common, affecting approximately 16% in those
20-59 yrs (237). Median concentrations over a 15 year period for the population have ranged between 466-483 pg/mL [344-356 pmol/L] with adult women in the same range (466-484 pg/mL) (17). Data from the 2004-2006 survey reported that the prevalence of deficiency was 2% in the general population and 4% in those ≥ 70 yr (221). A 2000 survey of older Canadians (> 50 y) just after mandatory fortification of FA found an average geometric mean of serum vitamin B\textsubscript{12} concentrations in women to be 332.5 pmol/L (238). The national Canadian Health Measures Survey (2007) found the geometric mean for serum vitamin B\textsubscript{12} in women aged 20-45 y to be 292 pmol/L with 5% classified as being deficient and 20.3% having a marginal status (61). More recently, Health Canada reported that, in 2009-2011, the mean serum vitamin B\textsubscript{12} concentration for the Canadian population (≥ 3 y) was 358 pmol/L and that ~4% of the Canadian population were deficient (239). Evidence suggests that the rate of deficiency may increase upwards to 10% as pregnancy progresses with the odds of being classified as deficient being 1.4 after 28 days of pregnancy (224).

2.6.2.3. Choline

There are two main sources of choline: dietary and the de novo synthesis pathway through the methylation of phosphatidylethanolamine to phosphatidylcholine (with the methyl group being donated from SAM) (217, 220). Women have a higher capacity than men for de novo choline synthesis as estrogen induces the expression of the phosphatidylethanolamine N-methyltransferase (PEMT) gene in the liver to make more choline to meet requirements (227). The PEMT pathway produces both choline and homocysteine; higher maternal homocysteine concentrations, with their attendant risk for preeclampsia, prematurity and low birth weight, can be the result of increased choline de novo synthesis if dietary intake is insufficient to meet demands (217, 226).

Choline-contributing compounds include free choline, and the esterified forms: glycerophosphocholine, phosphocholine, phosphatidylcholine (lecithin) and sphingomyelin; however the relative bioavailability of these compounds may differ and has not been completely established at this time (240). Choline is found in a wide variety of foods, both plant and animal, with liver, eggs, and wheat germ being the most concentrated sources (217). Human milk is also a rich source of choline compounds, with concentrations anywhere from 1-1.5 mmol/L with a
combination of phosphocholine, glycerophosphocholine, sphingomyelin, free choline and phosphatidylcholine (226).

Currently, there is a lack of evidence to establish an EAR for choline; therefore, choline has an adequate intake (AI) value. The AI for choline is based on preventing alterations in liver tissue, as indicated by elevated serum alanine aminotransferase concentrations (42). This information was derived from a single study done in males, using a single dose or deficiency to establish the outcome (42). Factors that can affect requirements include: gender (males as more sensitive to choline deficiency as females have a higher capacity for de novo synthesis); exercise (heavy exercise can reduce concentrations); bioavailability (which varies for the water-soluble and lipid-soluble forms of choline); the high demand for choline during pregnancy; and, metabolic variations (e.g. single nucleotide polymorphisms). None of these factors were considered when establishing the AI (42, 241). Requirements for women of childbearing years and pregnancy are shown in Table 2.3 as are population dietary intakes for the United States. Currently, there are no Canadian data examining choline intakes in the Canadian population on a national level or on which food sources are the primary contributors to the choline dietary intake. Choline is not found in the majority of supplements, including prenatal, making it critical to understand what choline food sources are being consumed in order to improve consumption of this critical nutrient during reproduction (226). The only prevalence of intakes below the AI on a population level that has been derived is based on extrapolation of the 2003-2004 NHANES data which found that mean choline intakes exceeded the AI for young children but 10% or less of the remaining population had intakes that met or exceeded the AI (242).

Table 2.5 summarizes data from the 2007-2008 NHANES (63), the first time choline was included in the dietary recall, which describes the top 5 dietary sources of choline for the U.S. population >1 y age.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Description</th>
<th>% Total</th>
<th>Cumulative</th>
</tr>
</thead>
</table>

Table 2.5. Dietary food sources for choline for U.S population¹
<table>
<thead>
<tr>
<th></th>
<th>Item</th>
<th>Choline</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eggs</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>Milk</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Meats: beef, pork, lamb, game</td>
<td>9</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Deli &amp; cured meats: ham, bacon, sausage etc</td>
<td>6</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>Poultry: chicken, turkey, duck etc</td>
<td>6</td>
<td>42</td>
</tr>
</tbody>
</table>

1 Data derived from (63).

2.6.3. Risk for Neural Tube and Other Birth Defects

There is a growing body of evidence suggesting that derangements in 1-carbon metabolism during the periconceptual period (~ 6 months around conception) are linked to adverse outcomes such as reproductive failure and birth defects (102). The evidence for folate is well-established (see Section 2.2.3.1) and there is a growing body of evidence for other critical nutrients in one-carbon metabolism, primarily for B₁₂ and choline. (See Table 2.6). Limited evidence suggests that B₆ is involved with NTD risk, but potentially in conjunction with other nutrients such as riboflavin, vitamins C & E, B₁₂ and choline (243-247). There is limited animal evidence looking at other methyl vitamins, but one animal study (243) looked at the effects of pyridoxine deficiency (0.1 mg pyridoxine) vs control (1 mg pyridoxine) in Sprague-Dawley rats during the first 20 days of gestation. They found a higher incidence of digital defects (48 vs 0), cleft palate (20 vs 0), omphalocele (12 vs 0) and exencephaly (6 vs 0) in those animals on the deficient diet (243). With vitamin B₁₂, the evidence of a protective effect against NTD risk with increasing maternal serum B₁₂ concentrations is relatively consistent (247-251), but not so for other congenital midline or syndrome defects where B₁₂ seems to have no effect (252). The maternal dietary intake studies for choline are inconclusive, with some suggesting that higher intakes of periconceptual choline have a protective effect against NTD, congenital diaphragmatic hernia and heart defects (253-255), while others show no effect with dietary choline intake (244). One animal study (254) compared the effects of a choline deficient diet (0.3 mg/kg), a riboflavin deficient diet (1 mg/kg) to a control diet (FA= 2 mg/kg, choline = 2.5 mg/kg, B₂ = 6 mg/kg) in MTHFR (+/+ ) and MTHFR (+/-) mice given for 6 weeks prior to mating. They found more delayed embryos in the choline and B₂ deficient diets compared to controls (15.44 vs 17.83 vs
8.65, respectively) in the MTHFR (+/-) mice and an increase in the percentage of heart defects for the choline and B2 deficient diets compared to controls (0.90 vs 1.20 vs 0.30, respectively) in the MTHFR (+/+ ) mice (254). A couple of studies used maternal serum concentrations and showed contradictory results with choline status depending on the defect; Shaw et al. (256) showed a strong linear association with a 76% reduction in NTD risk for every unit increase in choline intake and in a second study, Shaw et al. (246) demonstrated that high intakes of choline were associated with almost double the odds of having cleft lip/cleft palate. While limited, the evidence does suggest that dysfunction in one carbon metabolism from the central nutrients during crucial periods of neural development can increase the risk of neural tube or other birth defects.

2.7 SUMMARY

The primary role of 1-C metabolism is to provide carbon units for nucleotide synthesis and biological methylation. Dysregulation, either by genetic factors or from inadequate nutrient status of key nutrients such as folate, vitamin B12, vitamin B6 and choline could have adverse health effects in both the mother and offspring so an adequate status of all nutrients is crucial during the reproductive years to reduce the incidence of birth defects. Further, there is evidence that dysregulation can occur from an imbalance in nutrient status, either deficiencies or abundance of a single nutrient. A better understanding of how various factors including the state of pregnancy, FA supplementation, various socio-demographic, genetic and lifestyle factors can influence 1-C metabolism will further our understanding of why promoting adequate folate status is crucial to women of reproductive years. While the maintenance of folate status is necessary to promote normal function, it alone will not promote normal 1-C metabolism function. Rather, maintaining nutrient status for all the 1-C vitamins, including choline, B6 and B12 as well as folate, are necessary to promote normal function and prevent nutrient imbalances which are linked to increased risk of neural tube defects (NTD) and other birth defects. Understanding dietary choices of these nutrients, aside from supplement use, is needed to tailor health messages to promote the intake of these nutrients. This thesis then addresses these gaps in the literature and explores factors shown to affect the function of 1-C metabolism such as pregnancy, FA
supplementation, dietary sources and intake of methyl vitamins, genetics, and other socio-demographic and lifestyle influences in women of reproductive age.
**Table 2.6** Evidence examining NTD risk and other birth defects via other one carbon nutrients

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Study Design</th>
<th>Population</th>
<th>N</th>
<th>Intervention</th>
<th>Duration</th>
<th>Measure</th>
<th>Modifying Factors</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>B&lt;sub&gt;6&lt;/sub&gt;</td>
<td>case/control retrospective</td>
<td>10 states across United States</td>
<td>815-case, 6789-control</td>
<td>nutrient intake: the timing supplement intake (1st, 2nd-3rd trimester, none); and dietary intake</td>
<td>during pregnancy</td>
<td>rates of craniosynostosis and maternal dietary intake first trimester</td>
<td>FA fortified country</td>
<td>sagittal synostosis is ♣ with high intakes of B&lt;sub&gt;6&lt;/sub&gt; (≥ 2.7 mg) AOR = 0.4 (95% CI: 1.02, 0.6, P&lt;0.01); B&lt;sub&gt;2&lt;/sub&gt; (≥ 2.6 mg) AOR = 0.5 (95% CI: 0.3, 0.7, P&lt;0.01), Vit E (≥ 6.1 mg) AOR = 0.6 (95% CI: 0.4,0.9; P&lt;0.01), Vit C (≥ 154.1 mg) AOR = 0.7 (95% CI: 0.5, 1.1; P=0.02); increase in AOR for metopic synostosis with ↑ choline &amp; B&lt;sub&gt;12&lt;/sub&gt; intakes</td>
<td>(244)</td>
</tr>
<tr>
<td></td>
<td>case/control</td>
<td>France</td>
<td>77-case, 61-control</td>
<td>n/a (excluded if taking supplements)</td>
<td>n/a</td>
<td>Risk of NTD; by tertiles for maternal plasma folate, B&lt;sub&gt;6&lt;/sub&gt;, B&lt;sub&gt;12&lt;/sub&gt;, Hcy; RBC folate during pregnancy; and allele frequency of polymorphisms</td>
<td>non-FA fortified country</td>
<td>Cases had significantly ♣ RBC folate, B&lt;sub&gt;12&lt;/sub&gt; and B&lt;sub&gt;6&lt;/sub&gt; concentrations &amp; ↑ Hcy. In multivariate analysis, both RBC folate ↑ vs ↓ with OR= 0.25 (95% CI: 0.09-0.66, P=0.007) and B&lt;sub&gt;6&lt;/sub&gt; ↑ vs ↓ with OR= 0.38 (95% CI: 0.15-1.00, P=0.034) were predictors</td>
<td>(245)</td>
</tr>
<tr>
<td>B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>case/control</td>
<td>China</td>
<td>46-case, 44-control</td>
<td>n/a</td>
<td>Frequency of NTD; and maternal serum 5-methylTHF, total folate, B&lt;sub&gt;12&lt;/sub&gt;, Hcy, SAM, SAH were measured</td>
<td>non-FA fortified country</td>
<td>significantly lower log-transformed concentrations were related to increased risk for NTD for a) 5-methylTHF (9.32 vs 14383 nmol/L, P&lt;0.001), b) total folate (9.96 vs 16.25, P&lt;0.001) and c) B&lt;sub&gt;12&lt;/sub&gt; (83.24 vs 109.28, P=0.005)</td>
<td>(257)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>case/control</td>
<td>Texas-Mexico border</td>
<td>107-case, 275-controls</td>
<td>n/a</td>
<td>risk NTD; and quartiles of maternal serum folate and B&lt;sub&gt;12&lt;/sub&gt; concentrations</td>
<td>pre/post FA fortification years</td>
<td>There were significant inverse associations between quartiles for serum folate OR = 0.2 (95% CI: 0.08, 0.62; P=0.002); and B&lt;sub&gt;12&lt;/sub&gt; concentrations OR= 0.2 (0.07, 0.72; P= 0.012)</td>
<td>(250)</td>
<td></td>
</tr>
</tbody>
</table>
nested case/control 3 groups-1&3 NTD affected pregnancy, Group 2 -previous NTD but not current pregnancy

<table>
<thead>
<tr>
<th>Ireland</th>
<th>95 -case, 265-control</th>
<th>n/a (excluded if taking supplements)</th>
<th>n/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>risk NTD &amp; quartiles of maternal serum folate, B_{12}, RBC folate, country</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>frequency other congenital defects; and serum folate, B_{12}, Hcy, RBC folate concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median maternal RBC folate (271.2 vs 281.1, P=0.842) and Hcy did not differ between cases and controls for midline defects; B_{12} (326 vs 347.5, P=0.039) was significant for midline defects but not after adj for multiple testing</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Xinjiang Province, China</th>
<th>30-case, 60-control</th>
<th>n/a (excluded if taking B_{12} supplements)</th>
<th>n/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>freq NTD &amp; maternal serum folate, B_{12}, B_{6} and Hcy concentrations na/</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>maternal Hcy was significantly ↑ and plasma folate (9.7±8.1 vs 15 ± 8.1, P=0.005) &amp; B_{12} (181.3 ±107.7 vs 394.3 ±386.3, P&lt;0.001)were significantly ↓ between cases and controls while B_{6} was weakly significant (42 ±10.5 vs 48.7±16.5, P=0.051)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Texas | 34-case, 78-control | n/a |
| Dutch= 48 case, 62 control. 2 populations in: Holland & Texas |
| risk NTD & maternal serum B_{12}, folate, Hcy, DNA methylation levels of IGF-DMR, H19, KCNQ10T1, LEKR/CCNL, MTHFR, VANGL1 |
| 1) there was an inverse association between maternal B_{12} and NTD (AOR=0.38 (95% CI: 0.145-0.999, P=0.050) and 2) an association of DNA methylation of MTHFR CpG islands with NTD in Dutch group (47 vs 61, P<0.001) but not in the Texas group. |

Choline
case/control: California

424-case, 440-control

maternal periconceptual intakes of choline

FA fortified country

risk NTD and specific type of NTD & FA quartiles measures of choline & betaine intake

(95% CI: 0.27, 0.90) 2) NTD risk estimates lowest in those with diets rich (>75%ile) in choline (OR=0.17 (95% CI: 0.04, 0.76)).

1) strong inverse assoc between maternal choline intake (>498.46 mg/d) and risk NTD AOR= 0.49

population-based case/control: California

anencephaly= 141, spina bifida= 189, 625- control

FA containing supplements & quartiles of dietary intake in non supplement users

maternal dietary intake of folate, B2, B3, B6, B12, zinc, Vit C, Vit E, Vit A, choline & lutein

1) small non-significant effect from FA containing supplements and anencephaly OR= 0.8 (95% CI: 0.5, 1.3), or spina bifida OR=0.8 (95% CI: 0.6, 1.2) 2) low dietary intakes of folate (<294 µg) showed slight impact on spina bifida (OR=2.4, 95% CI: 1.1-5.4) but no effect seen with choline intake

nested case/control: California

80-case, 409-control

n/a

n/a

risk NTD, maternal FA supplement use maternal dietary intake of folate, B2, B3, B6, B12, zinc, Vit C, Vit E, Vit A, choline & lutein

1) inverse association between mean maternal B12 (292 vs 323, P=0.02), betaine (15.8 vs 16.7, P=0.05), and Cys (224 vs 234, P=0.02) while sig ↑ choline (3.09 vs 2.98, P=0.02) among cases ; 2) comparing ↑ & ↓ quartiles showed ↑ CLP risk for both low PPL (<35.25 nmol/L) AOR=1.8 (95% CI: 1.0-3.1), & high PPL (≥79.9) AOR=1.6 (95% CI: 0.9-2.9) and for high choline (<2.71 mmol/L) AOR=2.1 (95% CI: 1.2-3.50)

nested case/control: California

80-case, 409-control

n/a

n/a

risk NTD, maternal Hcy, MMA, folate, Vit B12, pyridoxal phosphate (PPL), pyridoxal, B2, choline, betaine, Met, Cys; deciles of choline concentrations

strong linear association total choline with ↓ NTD risk by decile: lowest (<2.49 mmol/L) AOR=2.4 (95% CI: 1.3-4.7); highest decile (>3.50 mmol/L) AOR=0.14 (95% CI: 0.02-1.0). The OR associated with 1 unit ↑ in choline is 0.24 (95% CI: 0.12-0.48). No effect for any other nutrients
<table>
<thead>
<tr>
<th>population-based case/control</th>
<th>California</th>
<th>377-case, 5008-control</th>
<th>maternal intake of 25 nutrients</th>
<th>maternal intake of 25 nutrients</th>
<th>year before pregnancy</th>
<th>FA fortified country</th>
<th>inverse association between dietary intake of a) choline (≥ 392.68 mg) AOR=0.6 (95% CI: 0.3-1.0), b) B vitamins, methionine B₆ (≥ 3.45 µg) AOR=0.3 (95% CI: 0.1-0.7), B₁₂ (≥ 10.30 µg) AOR=0.7 (95% CI: 0.4-1.4), methionine and CDH (255)</th>
</tr>
</thead>
</table>

1 Adjusted odds ratio, AOR; OR, odds ratio
3. RATIONALE AND OBJECTIVES

3.1. RATIONALE

The function of 1-C metabolism is for the provision of carbon units for nucleotide synthesis and biological methylation of a multitude of biological substrates. Dysregulation anywhere in the 1-C metabolic pathways could have adverse health effects in both the mother and offspring so an adequate status of all nutrients is crucial during the reproductive years. The synthesis and methylation pathways of 1-C metabolism are distinguished by interconvertable folate derivatives which differ by the one carbon substituent attached. Thus, maintaining adequate folate status during the reproductive years is critical, although evidence suggests that consideration of the other 1-C micronutrients, specifically choline, B₆ and B₁₂, is also required to maintain normal methylation function and reduce the incidence of birth defects. Currently there is limited evidence examining if pregnancy or the consumption of FA containing supplements in non-pregnant women are able to influence 1-C metabolism by altering the distribution of the folate derivatives in either the synthesis or methylation pathways.

The maintenance of folate status is necessary to promote normal function of 1-C metabolism. Various socio-demographic, genetic, and lifestyle factors including FA-containing supplement use, dietary intake, ethnicity, income, alcohol intake, genetics, education and age have all been linked to an increased risk of NTD in the years prior to FA fortification of the North American food supply. More recent post-fortification work found that: FA supplement use, ethnicity, income, and dietary intake are linked to RBC folate concentrations associated with NTD protection. However, this evidence is limited, as it comes from large epidemiological-based studies where only a few factors are tested at a time. Further, there is little information regarding detailed use of FA containing supplements, such as the minimum necessary dose and frequency of use needed to achieve NTD-protective RBC concentrations.

While the maintenance of folate status is necessary to promote normal function, it alone will not promote normal 1-C metabolism function. Rather, maintaining nutrient status for all the 1-C vitamins, including choline, B₆ and B₁₂ as well as folate, are necessary to promote normal function and prevent nutrient imbalances which are linked to increased risk of neural tube defects (NTD) and other birth defects. Supplement use of these nutrients is promoted as the simplest
method to ensure adequate nutrient status, but the majority of women of reproductive age do not use supplements at any given time. Thus, understanding key food sources of 1-C vitamins in women of reproductive age is needed to tailor health messages to promote the intake of these nutrients. Currently, the key food sources for these 1-C nutrients have never been systematically investigated at the national level in Canadian women. Further, there is no information about choline intakes in Canadian women or in the Canadian population as a whole.

Looking at the literature, it becomes apparent that the status of the key micronutrients involved in one-carbon metabolism can potentially be altered by various influences and has the potential to impact one-carbon metabolism. This then is the basis for this thesis: to explore how the status of the methyl nutrients can be influenced by various factors including pregnancy, FA supplementation, dietary sources and intake of methyl vitamins, genetics, and other socio-demographic and lifestyle factors that can influence women of reproductive age. (see Figure 3.1). On the diagram, those factors shown in black are the ones explored in this thesis, while those shown in gray are factors not explored in this thesis but have been shown in the literature to potentially influence individual nutrient status. Exploring how folate status is influenced is the primary focus of this thesis, but understanding if and how the nutrient status of the methyl nutrients folate, vitamins B₆ and B₁₂ and choline, can be altered is important given the interrelationship between these nutrients.

The following are the gaps we identified in the literature: 1) Do metabolic adaptations occur during pregnancy in order to accommodate the elevated folate requirements to support the rapid growth and development of the fetus and uteroplacental organs? Further, given the various physiological doses of folic acid women of childbearing years are exposed to, does folic acid supplementation cause any metabolic shifts in consequence of the repeated consumption of folic acid. 2) Would a more comprehensive list of factors (shown in epidemiological studies in the literature, both pre- and post-fortification of the food supply with folic acid, to influence the risk of neural tube defects), as well as more detailed information about these factors, better predict our ability to identify women with RBC folate concentrations associated with neural tube protective status? 3) Since the majority of women of childbearing years do not regularly consume multivitamins containing folic acid and the other methyl nutrients, what foods do Canadian women of childbearing years consume to contribute to their nutrient intake of folate,
vitamins B₆ and B₁₂, and choline? Further, what is the choline intake of Canadian women of childbearing years?

Our rationale then was to explore different factors including pregnancy, folic acid supplementation, genetic polymorphisms, dietary sources of methyl nutrients and other socio-demographic and lifestyle influences, all known to affect the folate status and other select micronutrients involved in 1-C metabolism in WCBY and lay the foundation to determine an appropriate multifaceted-approach to reduce the impact of any imbalances in 1-C metabolism.

3.2. OBJECTIVES

While we understand what folate does metabolically, there are still gaps in understanding the role of folate and one-carbon metabolism throughout the reproductive years. The overall objectives of this thesis then were to explore factors involved in one-carbon metabolism in women during their reproductive years, including pregnancy, FA supplementation, socio-demographic and other lifestyle factors, as well as dietary sources of folate and other methyl nutrients, such as vitamins B₆ and B₁₂, and choline, and to better understand how they relate to one-carbon metabolism.

The objectives for each study were:

1. The objectives of Study 1 were to determine if pregnancy or folic acid supplementation influences the distribution of the various folate forms in red blood cells.
2. The objectives in Study 2 were to determine what socio-demographic, genetic, lifestyle and dietary factors from a comprehensive list, enable us to better predict RBC folate concentrations associated with the reduction of neural tube defects.
3. The objectives in Study 3 were: a) to compile the predominant dietary sources of folate, vitamin B₆, vitamin B₁₂ and choline consumed by Canadian women of childbearing years; and, b) to calculate the choline intake in WCBY and determine the prevalence of intake below the AI.
Figure 3.1 Conceptual Diagram of Thesis. Diagram demonstrates how nutrient status of the methyl nutrients in one-carbon metabolism can be influenced by various factors. Factors discussed in thesis are highlighted in black while those not discussed but can affect nutrient status are highlighted in gray.
4. THESIS STUDY #1:

Neither folic acid supplementation nor pregnancy affects the distribution of folate forms in the red blood cells of women1-3

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RUNNING TITLE: RBC folate forms in women of reproductive age

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2 Author disclosures: B.A. Hartman, Z. Fazili, C.M. Pfeiffer and D.L. O’Connor have no conflicts of interest to disclose.
Supplemental Figure 1 is available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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Chapter 4 has been previously published:


4.1 ABSTRACT

Folate requirements are elevated during pregnancy to support growth and development. It is not known whether folate metabolism is altered to support increased DNA and RNA biosynthesis. Using a new sophisticated state-of-the-art liquid chromatography-tandem MS (LC-MS/MS) technique, the aim of this study was to investigate differences in RBC folate forms between pregnant and non-pregnant women and between non-pregnant women consuming different levels of supplemental folic acid (FA). The forms of folate in RBCs were used to explore potential shifts in folate metabolism during early erythropoiesis. Total RBC folate and folate forms (5-methylTHF, MeFox [oxidation product of 5-methylTHF], tetrahydrofolate [THF], 5-formylTHF and 5, 10-methenylTHF) were measured in four groups of women (n=26) including: pregnant women (PW, 30-36 wks gestation) consuming 1.0 mg of FA/d; non-pregnant women consuming 0 (NPW-0), 1.0 (NPW-1), and 5.0 mg (NPW-5) FA/d. The mean (±SD) RBC folate concentration of the NPW-0 group (890±530 nmol/L) was lower than the NPW-1 (1660±350 nmol/L) and NPW-5 (1980±570 nmol/L) groups assessed by microbiological assay (n=26, P<0.0022). No difference was found between the NPW-1 and NPW-5 groups. We detected 5-methylTHF (limit of detection [LOD] = 0.06 nmol/L) in all and THF (LOD = 0.2 nmol/L) in most women regardless of methylenetetrahydrofolate reductase genotype (C677T). A majority of women consuming FA supplements (PW, NPW-1, NPW-5) had detectable concentrations of 5,10-methenylTHF (LOD=0.31 nmol/L). However, there was no significant difference in the relative distribution of 5-methylTHF (83-84%), sum of non-methyl folates (0.6-3%) or individual non-methyl folate forms in RBCs across groups. We conclude that while FA supplementation in non-pregnant women increases RBC total folate content and the
concentration of individual RBC folate forms, it does not alter the relative distribution of folate forms. Similarly the distribution of RBC folate forms did not differ between pregnant and non-pregnant women. Trial registered at ClinicalTrials.gov (NCT01741077).
4.2 INTRODUCTION

Requirements for folate are elevated during pregnancy due to increased demand for purines and pyrimidines to facilitate rapid RNA and DNA biosynthesis; the transfer of one-carbon units via tetrahydrofolate (THF)\(^7\), 10-formylTHF and 5,10-methyleneTHF are key in these anabolic pathways (67). In a competing pathway, 5-methyltetrahydrofolate (5-methylTHF) facilitates remethylation of homocysteine to produce methionine which is then converted to S-adenosylmethionine (SAM), the universal methyl donor in the body (67). The metabolic changes that occur in pregnancy to accommodate elevated folate requirements are not fully understood.

Periconceptual folic acid (FA) supplementation and FA fortification of enriched cereal grain products have been mandatory in North America since 1998 and has resulted in a 26-47% reduction in neural tube defects (NTDs) (5, 6, 259). The Institute of Medicine (42) recommends that women able to become pregnant continue to consume 400 µg/d of FA from fortified foods and/or supplements, and at least 90% of North American women report taking a FA-containing prenatal vitamins during pregnancy (40, 113, 132). In Canada, available prenatal supplements contain either 1 or 5 mg FA. While the benefits of FA in the prevention of birth defects is acknowledged as a tremendous public policy success, some published accounts in the literature have associated FA supplementation during pregnancy with the risk of asthma, respiratory infection, wheeze, and central adiposity and insulin resistance in offspring; an equal number or more find no such association (34, 37, 260). An understanding of the physiological shifts in folate metabolism that are associated with pregnancy and or FA supplementation may improve

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\(^7\) Abbreviations used: DFE, dietary folate equivalents; FA, folic acid; LC-MS/MS, liquid chromatography tandem MS; MeFox, 4α-hydroxy-5-methylTHF; C677T MTHFR, methylenetetrahydrofolate reductase; MVM, multivitamin and mineral supplement; NPW-0, non-pregnant women not consuming a FA supplement; NPW-1, non-pregnant women consuming a 1 mg/d FA supplement; NPW-5, non-pregnant women consuming a 5 mg/d FA supplement; NTD, neural tube defect; PW, pregnant women; SAM, S-adenosylmethionine; THF, tetrahydrofolate.
our understanding of how maternal FA supplementation may influence the health of offspring both short and long-term. Physiological shifts in folate metabolism including in RBCs (reflecting folate metabolism earlier in their genesis) are well described in the literature secondary to dietary deficiencies, environmental exposures and disease (45-49, 73, 261). We hypothesize a shift toward non-methylated forms of folate (e.g. THF, formylTHF etc) during pregnancy may be one of the mechanisms to accommodate increased DNA and RNA biosynthesis.

There are several studies on the forms of folate in serum and RBCs of non-pregnant adults. It is estimated that 85-100% and 0-11% of the naturally occurring folate in serum is in the 5-methylTHF and THF form, respectively (156, 159, 262). The distribution of RBC folate forms reported in both males and pre-menopausal women suggests that 87-100% of the RBC folates are in the 5-methylTHF form with a small percentage as THF (0-15%) and 5, 10-methenylTHF (0-2%) (151, 155, 157, 158, 160). Currently, the distribution of folate forms in RBCs of pregnant women and in non-pregnant women consuming various doses of FA supplements has largely been unexplored.

The purpose of this study was to compare the distribution of folate forms in RBCs of pregnant and non-pregnant women and among non-pregnant consuming three levels of supplemental FA (0, 1 or 5 mg/d) using a new and improved state-of-the-art liquid chromatography-tandem MS (LC-MS/MS) technique (144, 145, 263). Understanding how pregnancy and FA supplementation influence the intracellular distribution of folate may help elucidate any metabolic adaptations that occur during reproduction or at pharmacological doses of FA.

4.3 METHODS
Subjects

The study protocol was approved by the Research Ethics Board of The Hospital for Sick Children and informed written consent secured. Women were excluded from participation if they had a pre-existing medical condition or were consuming medications known to interfere with folate absorption or metabolism. Thirty-two healthy, non-smoking women (20 to 41 y of age) were recruited from staff and visitors at the Hospital for Sick Children in Toronto Canada between January and October 2008. Folic acid fortification of white wheat flour (150 µg/100 g flour) and grain products labeled enriched became mandatory in Canada in 1998 (264). Eight of the women were pregnant between 30 to 36 weeks gestation (PW) and were consuming 1 mg/day FA as part of a multivitamin and mineral prenatal (MVM) supplement. The 24 remaining women were all non-pregnant/non-lactating (NPW) and were evenly divided between: did not consume a FA-containing MVM supplement (NPW-0); consumed 1 mg/d FA as part of a MVM supplement (NPW-1) and consumed 5 mg/d FA as part of a MVM supplement (NPW-5). All MVM supplements (Prenatal MVM, Life Brand; Materna, Nestle; Pregvit, Duchesnay; Prenatal Jamieson) included a source of vitamins B₆ and B₁₂. Women in the NPW-1 and NPW-5 groups were co-recruited with another study where supplements were provided for 30 weeks verified by pill counts (265).

Study design and methods

Women participating in this cross-sectional study fasted overnight and refrained from taking their usual supplement (if using) on the morning of their study visit. During the visit, subjects filled out a socio-demographic and supplement use questionnaire and the validated Block Folic Acid/Dietary Folate Equivalents Screener (NutritionQuest, Berkeley, CA) (266). Venous blood samples were collected into EDTA lined tubes and processed within two h of
collection. Aliquots of whole blood (100 µl) for determination of total RBC folate concentration by microbial assay were diluted 10-fold with ascorbic acid (0.057 mol/L), mixed, incubated for 30 min at 37°C, and then frozen (142). Remaining whole blood was centrifuged (850 x g for 15 min at 4°C) to separate plasma and RBCs. The buffy coat was used for C677T methylenetetrahydrofolate reductase genotype (MTHFR) genotyping (267). Sodium ascorbate solution (0.057 mol/L) was added to plasma samples prior to storage. Packed RBCs used for the later determination of folate forms by LC-MS/MS were washed twice and re-suspended in an equal volume of NaCl (0.154 mol/L). The suspension was then diluted 20-fold with ascorbic acid solution (0.028 mol/L, pH 4.2) and charcoal-treated human serum folate conjugase was added (100 µl human serum to ~100 nmol folate); this hemolysate was incubated for 1 h at 37°C (162). Aliquoted whole blood and, RBC hemolysates and plasma samples were stored at -80°C.

Total folate concentrations in whole blood hemolysate and plasma were measured by microbiological assay as described by Molloy and Scott (142) using non-chloramphenicol-resistant Lactobacillus rhamnosus (ATCC7649, American Type Tissue Culture Collection, Manassas, VA) with folic acid to generate the standard curve. RBC folate content was calculated using the analyzed whole blood folate concentration minus the plasma folate concentration corrected for hematocrit. A whole blood standard from the National Institutes for Biological Standards and Control (code 95/528, Hertfordshire, United Kingdom) with a certified folate content of 29.5 nmol/L was used in every assay. The overall inter-assay coefficient of variation for the whole blood folate standard was 7.4% with a mean value of 28.2 nmol/L.

The concentrations of folate forms in RBCs (5-methylTHF, MeFox [pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF], THF, 5-formylTHF and 5,10-methenylTHF) were determined using LC-MS/MS (144, 174, 263). RBC hemolysates (150 µL) were mixed with
ammonium formate buffer and amended with a mixture of $^{13}$C5-labeled folate internal standards. Sample clean-up was performed using a 50-mg phenyl solid phase extraction (SPE) 96-well plate (Bond Elut 96; Agilent Technologies) and an automated 96-probe SPE system (Caliper-Zephyr; Perkin Elmer) (174). Samples were eluted from the SPE plate with an organic elution buffer containing ascorbic acid and analyzed overnight by LC-MS/MS in positive ion mode using electrospray ionization on a Sciex API 5500 triple-quadrupole MS system (Applied Biosystems) coupled to a HP1200C LC system (Agilent Technologies). Chromatographic separation was achieved using a Luna C-8 analytical column (Phenomenex) with an isocratic mobile phase and a total run time of 7 min (174). Three whole blood hemolysate bench quality control (QC) pools were analyzed in duplicate in every run, bracketing the study samples. The between-run imprecision ($n = 5$ days) for 3 QC levels (2 levels for THF, 5-formylTHF and 5,10-methenylTHF) was 2.2–3.0% for 5-methylTHF (20.6–37.1 nmol/L), 3.3–5.7% for MeFox (3.47–6.40 nmol/L), 4.6–8.2% for THF (4.53–8.19 nmol/L), 5.5–5.6% for 5-formylTHF (2.97–5.60 nmol/L) and 3.3–6.9% for 5,10-methenylTHF (4.84–9.75 nmol/L). The limit of detection (nmol/L hemolysate) values were: 0.06 (5-methylTHF), 0.08 (MeFox), 0.2 (THF), 0.2 (5-formylTHF) and 0.31 (5,10-methenylTHF).

**Statistical analysis**

Prior to statistical analysis, RBC folate data were log-transformed. A Pearson product-moment correlation coefficient was computed to assess the relationship between RBC total folate concentrations determined by microbiological assay and LC-MS/MS. Mean differences in RBC total folate concentrations, concentration of different folate forms and the % distribution of different forms were determined by ANOVA. When a statistically significant difference was found, this was followed by pair-wise comparisons using the Tukey-Kramer method.
indicated, individuals homozygous for the C677T MTHFR allele were removed from the analysis as it is known that these individuals accumulate non-methyl folate forms (151, 152, 154). Statistical tests were performed using SAS (version 9.1, SAS Institute Inc., Cary, NC) and a P-value of < 0.05 was considered statistically significant. Values in the text are expressed as mean ± SD.

4.4 RESULTS

As illustrated in Figure 4.1, 32 women were enrolled in the study; however a few sample vials cracked during freezer storage (n=5; 1 PW, 2 NPW-0, 2 NPW-1) and 26 samples were available for analysis. All women had a minimum of some college education, and there were no differences in age, or education among the groups; however there were differences in income and alcohol use (Table 4.1). There was no difference in dietary folate intake (naturally occurring and FA added as a fortificant) among groups and all women reported consuming folic acid fortified foods. Only one subject in the NPW-0 consumed a vitamin or mineral supplement and this consisted of calcium and vitamin D only. Except for one subject in the NPW-5 group who reported consuming her supplement every other day, all women consuming a supplement reported doing so daily. Mean duration of supplement use was longer in the PW group (46 ± 8 wks) compared to NPW-1 and NPW-5 groups (30 ± 0 wks, P<0.001).

RBC folate concentrations determined by microbiological assay or LC-MS/MS (sum of folate forms) methods were strongly correlated (n=26, r=0.91, P<0.0001). Mean RBC folate concentrations did not differ between PW and NPW-1 as assessed by microbiological assay or LC-MS/MS (Figure 4.2). NPW-0 had lower RBC folate concentrations than either NPW-1 or NPW-5 women as assessed by LC-MS/MS and lower RBC folate concentrations than NPW-5 assessed by
microbiological assay. There was no significant difference in mean RBC folate concentrations between NPW-1 and NPW-5, measured by either microbiological assay or LC-MS/MS.

Distributions of folate forms in RBCs and C677T MTHFR genotype for each participant are found in Supplemental Figure 4.1. Mean group values are presented in Table 4.2 with individuals homozygous for the MTHFR C677T allele excluded. No differences in 5-methylTHF or MeFox were found between PW and supplemented NPW groups, expressed as a concentration or percent distribution. Concentration of 5-methylTHF in RBCs of NPW-0 women was less than NPW-5 women but did not differ from NPW-1 women. No differences among NPW-0, NPW-1 and NPW-5 women existed in either 5-methylTHF or MeFox as a percent of total folate.

There were no statistically significant group differences in the sum of non-methyl folates or in the individual non-methyl folate forms expressed as a concentration or as a proportion of total folate among PW and NPW women consuming 1 mg/d FA. The concentration of the sum of all non-methyl folates (THF, 5-formylTHF and 5,10-methenylTHF) of NPW-0 women was less than in the NPW-1 and NPW-5 women groups as was the concentration of THF. Nonetheless no differences among NPW-0, NPW-1 and NPW-5 women existed in the sum of all non-methyl folate or in the individual non-methyl forms as a percent of total RBC folate.

As expected, we found THF and 5,10-methenylTHF in RBCs of women who were C677T MTHFR homozygous regardless of supplementation status (Supplemental Figure 4.1); however, we also found measurable concentrations of THF (>0.2 nmol/L hemolysate) and 5,10-methenylTHF (>0.31 nmol/L hemolysate) in RBCs of women who had both a wild type and heterozygous genotype. Three of 5 NPW taking 0 mg FA had measurable concentrations of THF and one had 5, 10-methenylTHF. Even after excluding C677T MTHFR homozygotes, most of
the PW taking 1 mg FA (4/6) had measurable concentrations of 5-formylTHF in RBCs whereas there was only one of four women in the NPW-1 group who had measurable concentrations.

4.5 DISCUSSION

There is strong evidence in the literature that folate requirements increase during pregnancy to support rapid fetal and uteroplacental growth (3, 92) and yet the changes that occur to support this rapid rate of anabolic activity have not been fully explored. In the present study we did not find any difference in the distribution of folate forms in RBCs between PW and NPW-1 groups suggesting that there is little evidence folate is preferentially utilized for purine and pyrimidine synthesis for erythropoiesis in pregnancy. If a physiological response in folate metabolism occurred to accommodate increased DNA/RNA synthesis, we would anticipate a shift in folate metabolism from re-methylation of homocysteine to methionine to purine and pyrimidine biosynthesis resulting in a corresponding increase in non-methyl folates forms.

To our knowledge this is the first time that the RBC folate forms between pregnant women and non-pregnant women or non-pregnant women consuming different levels of supplemental FA have been directly compared. As far as we are aware, only two groups have examined the distribution of folate forms in blood during pregnancy. Obeid et al. (161) reported that the total serum folate, 5-methylTHF, 5-formylTHF and THF concentrations of FA-supplemented (400 µg/d) pregnant women immediately prior to delivery were greater than pregnant women not consuming a FA supplement. However, like the results of the current study, the distribution of the different folate forms as a relative percentage of total folate remained unchanged between the FA supplemented pregnant women and the non-supplemented pregnant women. In FA-supplemented pregnant women, 92.9%, 0.94% and 8.3% of total serum folate
was in the 5-methylTHF, formylTHF and THF form, respectively, compared to 85.4%, 0.95% and 11.7% among unsupplemented pregnant women.

Houghton et al. (162) reported that RBC folate forms of pregnant women (equal number of women with C/C, C/T and T/T C677T MTHFR genotypes), consuming 1 mg/d FA, contained a significant proportion of RBC folates in the THF form (55-59.5%). These data differ from those in the present study where only a small percentage of total folates were found as THF regardless of whether women were pregnant or were supplemented with FA. In the former study, it was postulated that the elevated THF reflected cellular uptake of high concentrations of circulating unmetabolized FA early in the erythroid lineage or alternatively reflected the elevated concentration of high-affinity binding proteins during pregnancy which are believed to protect labile THF from degradation (268, 269). Results from the current study suggest, however, that the elevated THF concentrations in the latter study likely reflected an analytical aberration, potentially resulting from folate form interconversions during analysis, i.e., between 5,10-methylenethiol and THF, causing an overestimation of THF (140, 162).

There is considerable evidence in the literature suggesting that physiological shifts in folate metabolism do occur in response to certain conditions such as: dietary deficiencies, environmental exposures, and abnormal anabolic conditions such as cancer. For example, dietary deficiencies of methionine (in kwashiorkor), folate or vitamin B₁₂ result in a reduction of SAM and a corresponding rise in 5-methylTHF due to the reduced activity of methionine synthase which converts 5-methylTHF to THF (45). SAM concentrations play an important regulatory role in folate metabolism and low levels stimulate MTHFR activity to shunt folate to 5-methylTHF and divert folate away from purine and pyrimidine biosynthesis (45). Correction of methionine, folate or vitamin B₁₂ deficiency will reduce 5-methylTHF, increase SAM and hence
increase formylTHF concentrations (substrate for purine biosynthesis) and conversion of 5,10-methenylTHF to thymidylate (for DNA biosynthesis) (46). Second, it was reported that higher proportions of formylTHF and THF and lower proportions of 5-methylTHF are found in colorectal tumors compared to normal mucosa with no correlation to RBC folate concentration (48). This shift in folate forms was accompanied by a decrease in global DNA methylation, thought to be an important mechanism in the pathogenesis of colorectal cancer (261). In the current study, we did not find evidence that RBC folate, reflecting metabolism earlier in erythropoiesis, was shunted toward purine and pyrimidine biosynthesis during pregnancy; however we can’t discount the possibility this may occur in other tissues.

Limitations to this study include the small sample size; however results obtained using our new and sophisticated LC-MS/MS technique indicate that differences between the relative distribution of RBC folate forms, our main outcome, was small. To determine the small effect size observed between PW and NPW-1 women, for example, for % 5-methylTHF, % sum of non-methylTHFs and % THF would require sample sizes of 72, 512 and 364, respectively. Whether the small differences noted are clinically relevant and warrant larger studies is uncertain. Second, recent evidence suggests that it may take longer than one complete turnover of all RBCs (120 days or 17 wk) to reach a new steady state RBC folate concentration after commencement of FA supplementation (167, 270). PW in our study had consumed FA supplements longer than NPW women (46 ± 8 vs 30 ± 0 wk, P<0.001) which leaves open the possibility that differences in the main outcome (relative distribution of RBC forms) could have been due to differences in the duration of FA supplementation. As no differences were found, we do not believe this limitation biased our study conclusions. Finally, it should be noted that all women in this study, including those in the NPW-O group were consuming folic acid fortified
foods. It is unclear whether the results presented herein can be generalized to reproductive-age women in countries where FA fortification is not present.

In conclusion, results of this study suggest that there is little evidence that folate is being preferentially utilized early in erythropoiesis for purine and pyrimidine synthesis during pregnancy as we found no increase in the proportions of non-methyl folate forms. Furthermore, there is little evidence that FA supplementation at high levels (1 and 5 mg/d) alters the distributions of the folate forms in RBCs. Given our small sample size, it is unclear whether our results can be generalized to all reproductive-age women, particularly those not consuming folic acid fortified foods with baseline RBC folate concentrations lower than reported herein. The data presented herein do, however, provide the basis for further investigation.

Acknowledgments

The authors wish to thank Dr. Gideon Koren and Dr. Patricia Nguyen for allowing us to co-recruit women from their on-going folic acid supplementation trial.

B.A.H., and D.L.O., conceived the study; B.A.H. conducted the research, performed the microbiological assay and statistically analyzed the data. Z.F and C.M.P performed the LC-MS/MS analysis. BAH and DLO drafted the manuscript; All authors contributed to the critical revision of the manuscript for important intellectual content and read and approved the final version.
Figure 4.1. Disposition of study subjects and availability of samples for analysis. LC-MS/MS, liquid chromatography tandem MS; C677T MTHFR, methylenetetrahydrofolate reductase C677T genotype; PW, pregnant women consuming 1 mg/d FA as a supplement; NPW-0, non-pregnant women not consuming a FA supplement; NPW-1, non-pregnant women consuming 1 mg/d FA as a supplement; NPW-5, non-pregnant women consuming 5 mg/d FA as a supplement.
Figure 4.2: Mean RBC total folate concentrations in pregnant women (PW) consuming 1 mg /d folic acid and non-pregnant women consuming 0 (NPW-0), 1 (NPW-1) or 5 (NPW-5) mg/d FA as determined by microbiological assay or liquid chromatography tandem MS. Women homozygous for the C677T MTHFR genotype were not included in these analysis. Values are means ± SD; PW (n=6), NPW-0 (n=5), NPW-1 (n=5), NPW-5 (n=7). Within an assay, means without a common letter differ, P< 0.05.
### TABLE 4.1  Characteristics of pregnant and non-pregnant female participants consuming different amounts of supplemental folic acid

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<th>PW (n=7)</th>
<th>NPW-0 (n=6)</th>
<th>NPW-1 (n=6)</th>
<th>NPW-5 (n=7)</th>
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<td>31 ± 4</td>
<td>36 ± 6.6</td>
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<td>Ethnicity, n (%)</td>
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<tr>
<td>White</td>
<td>6 (85)</td>
<td>2 (33)</td>
<td>5 (83)</td>
<td>2 (28)</td>
<td></td>
</tr>
<tr>
<td>Non-white</td>
<td>1 (15)</td>
<td>4 (67)</td>
<td>1 (17)</td>
<td>5 (72)</td>
<td></td>
</tr>
<tr>
<td>Income, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0207</td>
</tr>
<tr>
<td>&lt; 60 000</td>
<td>4 (57)</td>
<td>6 (100)</td>
<td>5 (83)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>≥ 60 000</td>
<td>3 (43)</td>
<td>0 (0)</td>
<td>1 (17)</td>
<td>5 (71)</td>
<td></td>
</tr>
<tr>
<td>Education, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>College or university</td>
<td>4 (57)</td>
<td>1 (17)</td>
<td>2 (33)</td>
<td>3 (43)</td>
<td></td>
</tr>
<tr>
<td>&lt; baccalaureate</td>
<td>3 (43)</td>
<td>5 (83)</td>
<td>4 (67)</td>
<td>4 (57)</td>
<td></td>
</tr>
<tr>
<td>Alcohol use, n (%)</td>
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<td></td>
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<td>0.0396</td>
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<tr>
<td>Don’t drink</td>
<td>7 (100)</td>
<td>2 (33)</td>
<td>1 (17)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>≤ 1/wk</td>
<td>0 (0)</td>
<td>1 (17)</td>
<td>2 (33)</td>
<td>4 (57)</td>
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<tr>
<td>≥ 2/wk</td>
<td>0 (0)</td>
<td>3 (50)</td>
<td>3 (50)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>C677T MTHFR genotype, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.47</td>
</tr>
<tr>
<td>C/C</td>
<td>1 (14)</td>
<td>3 (50)</td>
<td>1 (20)</td>
<td>4 (67)</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
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<td>T/T</td>
<td>1 (14)</td>
<td>1 (17)</td>
<td>1 (12.5)</td>
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<td></td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary folate intake, µg DFE/d</td>
<td>480 ± 230</td>
<td>590 ± 140</td>
<td>390 ± 80</td>
<td>480 ± 190</td>
<td>0.33</td>
</tr>
<tr>
<td>Plasma folate concentrations, nmol/L&lt;sup&gt;2&lt;/sup&gt;</td>
<td>80 ± 13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>46 ± 33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54 ± 9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>92 ± 34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0095</td>
</tr>
<tr>
<td>RBC folate concentrations, nmol/L&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2130 ± 630&lt;sup&gt;a&lt;/sup&gt;</td>
<td>890 ± 530&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1660 ± 350&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1980 ± 570&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0022</td>
</tr>
</tbody>
</table>

<sup>1</sup> Values are means ± SD, or n (percentages) n=26. Means without a common letter differ P < 0.05. DFE, dietary folate equivalents; FA, folic acid; MTHFR, methylenetetrahydrofolate reductase; PW, pregnant women taking 1 mg/d of FA as a supplement; NPW, non-pregnant women taking 0, 1 or 5 mg/d of FA as a supplement.

<sup>2</sup> Plasma and RBC folate concentrations measured by microbiological assay
<table>
<thead>
<tr>
<th>Folate Form</th>
<th>PW (n=6)</th>
<th>NPW-0 (n=5)</th>
<th>NPW-1 (n=4)</th>
<th>NPW-5 (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C677T MTHFR C/C</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>C677T MTHFR C/T</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5-methylTHF, nmol/L</td>
<td>1600 ± 230&lt;sup&gt;a&lt;/sup&gt;</td>
<td>160&lt;sup&gt;b&lt;/sup&gt;</td>
<td>225&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>420&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0002</td>
</tr>
<tr>
<td>% Total folate</td>
<td>83.5 ± 2.2</td>
<td>83.2 ± 0.9</td>
<td>82.4 ± 0.8</td>
<td>84.3 ± 1.1</td>
<td>0.25</td>
</tr>
<tr>
<td>MeFox, nmol/L</td>
<td>261 ± 53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>198 ± 27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>240 ± 64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0002</td>
</tr>
<tr>
<td>% Total folate</td>
<td>13.5 ± 1.1</td>
<td>15 ± 0.6</td>
<td>15 ± 1.6</td>
<td>14.4 ± 1.5</td>
<td>0.21</td>
</tr>
<tr>
<td>Sum of non-methyl folate, nmol/L&lt;sup&gt;2&lt;/sup&gt;</td>
<td>48 ± 50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36 ± 33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19 ± 11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0303</td>
</tr>
<tr>
<td>% Total folate</td>
<td>3 ± 2.7</td>
<td>0.6 ± 0.7</td>
<td>2.4 ± 0.2</td>
<td>1.1 ± 0.6</td>
<td>0.24</td>
</tr>
<tr>
<td>THF, nmol/L</td>
<td>26 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24 ± 20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0408</td>
</tr>
<tr>
<td>% Total folate</td>
<td>1.3± 0.9</td>
<td>0.5± 0.5</td>
<td>1.6± 1.2</td>
<td>0.7± 0.4</td>
<td>0.47</td>
</tr>
<tr>
<td>5-formylTHF, nmol/L&lt;sup&gt;3&lt;/sup&gt;</td>
<td>7 ± 4</td>
<td>0 ± 0</td>
<td>5 ± 0</td>
<td>0 ± 0</td>
<td>n/a</td>
</tr>
<tr>
<td>% Total folate</td>
<td>0.3 ± 0.25</td>
<td>0 ± 0</td>
<td>0.3 ± 0</td>
<td>0 ± 0</td>
<td>n/a</td>
</tr>
<tr>
<td>5,10-methenylTHF, nmol/L</td>
<td>26 ± 38</td>
<td>1 ± 3</td>
<td>11 ± 11</td>
<td>5 ± 5</td>
<td>0.67</td>
</tr>
<tr>
<td>% Total folate</td>
<td>1.4± 2.1</td>
<td>0.2± 0.3</td>
<td>0.7± 0.7</td>
<td>0.3± 0.2</td>
<td>0.61</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are the mean ± SD. Means without a common letter differ, P < 0.05. C677T MTHFR, C677T methylenetetrahydrofolate reductase genotype; PW, pregnant women consuming 1 mg/d FA in supplement form; NPW-0, non-pregnant women not consuming FA in supplement form; NPW-1, non-pregnant women consuming 1 mg/d FA in supplement form; NPW-5, non-pregnant women consuming 5 mg/d FA in supplement form; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF.

<sup>2</sup>Sum of THF + 5-formylTHF + 5,10-methenylTHF

<sup>3</sup>Insufficient cell size for analysis of 5-formylTHF.
Supplemental Figure 4.1: Distribution of folate forms in RBCs of pregnant women (PW) and non-pregnant (NPW) consuming 0, 1 or 5 mg folic acid (FA) per day as a supplement as assessed by liquid chromatography-tandem MS. Figure 1A shows the breakdown for 5-methylTHF, MeFox, and the sum of non-methyl folate forms while Figure 1B shows the detail for non-methyl folate forms (THF, 5-formylTHF, and 5,10-methenylTHF). Crosses, asterisks and number signs denote subjects who are wild type (C/C), heterozygous (C/T) and homozygous (T/T) for the C677T methylenetetrahydrofolate reductase (MTHFR) allele.
An enhanced list of sociodemographic, dietary and lifestyle factors does not identify women with RBC folate concentrations associated with protection against neural tube defects\textsuperscript{1,2}

**RUNNING TITLE:** Indicators of RBC folate status

**AUTHOR LIST FOR INDEXING:**

\textsuperscript{1} From the Research Institute, Physiology and Experimental Medicine, The Hospital for Sick Children, Toronto, ON.

\textsuperscript{2} Supported by CIHR Operating Grant 218776. Funding entity did not have a role in the design, implementation, analysis or interpretation of the data.

*To whom correspondence should be addressed.

\textsuperscript{6} Abbreviations used: FA folic acid, MTHFR methylenetetrahydrofolate reductase, NTD neural tube defect, WCBY women of childbearing age
5.1. ABSTRACT

**Background:** Increased maternal folate status is associated with a reduction in neural tube defects (NTD). Socio-demographic and lifestyle indicators collected as part of the Canadian Health Measures Survey account for < 30% of the variation in RBC folate concentrations but did not include information on other indicators associated with NTD risk or detailed information in folic acid (FA) supplement use. Using an expanded list of potential indicators of folate status, our aim was to re-evaluate the socio-demographic, dietary, genetic, and lifestyle indicators associated with NTD protective concentrations of RBC folate among women of child-bearing years (WCBY), with the long term goal to develop a tool to be used by clinicians to identify women with NTD-protective folate status.

**Method:** RBC folate concentrations, C677T MTHFR genotype, dietary folate intake (Block Folic Acid/Dietary Folate Equivalents Screener), and detailed questions on: folate dietary sources; FA and B12 supplement intake; education; ethnicity; income; birth status; age, smoking; and alcohol use were collected from 101 WCBY. The relationships between RBC folate and variables of interest were assessed using multiple linear and logistic regressions, with the predictive ability of different models determined using area under the ROC curve.

**Results:** Mean RBC folate was 850 ± 265 nmol/L and 58% had concentrations ≥ 906 nmol/L. Increasing FA supplement dose was associated with an improved odds (OR 1.9, 95% CI 1.1, 3.2) of optimal folate status (≥ 906 nmol/L), while using supplements ≥ twice a week was associated with an odds of 12.6 (95% CI 2.2, 70.2). Women who consumed alcohol less than once a week were 66% more likely to have sub-optimal folate concentrations (p=0.0546). Overall, the final models containing variables for FA supplement dose and alcohol intake (controlling for ethnicity) effectively discriminate a folate status ≥ 906 nmol/L, 73% of the time.
Conclusions: We conclude that a comprehensive list of variables does not improve the prediction of folate status in WCBY. This work suggests that focusing on dose and frequency of FA supplement use while asking information about alcohol intake provides the best method to predict RBC folate status in WCBY.
5.2. INTRODUCTION

Adequate folate status during reproduction is required not only to reduce the incidence of neural tube defects (NTD)\(^6\) but for adequate growth and development of fetal and maternal tissue (67, 92). Folic acid (FA) fortification of white wheat flour and select grain products labelled as enriched, and periconceptional FA supplementation have reduced the incidence of NTD by approximately 26-47% in North America (6, 105, 259). The Institute of Medicine recommends that all women of childbearing years (WCBY) consume 400 µg of synthetic FA from supplements or fortified foods (or both) in addition to a varied diet for adequate folate status (42). Despite these recommendations, approximately 22% of North American WCBY have RBC folate concentrations below 906 nmol/L, a frequently used cut-off of maternal folate status associated with the lowest risk of folate-mediated NTD (9, 54).

There is a growing body of evidence suggesting a U-shaped risk effect with folate status. Risk for NTD and other birth defects such as cleft lip and palate, megaloblastic anemia, birth weight, stroke, cancer are all associated with inadequate folate status (4, 271, 272). More controversial is concern that provision of super-physiological concentrations FA may exacerbate the effects of suboptimal B\(_{12}\) intake, reduce the efficacy of antifolate drugs and increase the risk of certain cancers (21, 42, 211). Further, evidence, albeit conflicting, suggests that maternal FA supplementation impacts fetal programming and may influence central adiposity, insulin resistance, and respiratory issues in offspring (33, 37, 273).

The most direct approach to assess whether WCBY have optimal circulating concentrations of folate to reduce folate-mediated NTD risk and avoid prescribing unnecessarily high intakes of FA, would be to measure RBC folate concentrations. As this approach would be expensive to implement on a population level for all WCBY (50) and there is no established cut-
off for NTD reduction of risk at this time, we are interested in exploring alternative methods to predict if a woman is more likely to have a folate status associated with NTD protection.

Prior to FA fortification of the food supply, factors such as adolescence, low socio-economic status, low consumption of folate-rich foods, smoking, alcohol use, low compliance with FA containing supplements and maternal obesity were all associated with an increased risk of NTD (51-53). A more recent post-fortification study, using data from the 2007-2009 Canadian Health Measures Survey, found that high income, maternal age, use of FA and B₁₂ supplements, not being born in Canada and higher fruit and vegetable intake were associated with RBC folate concentrations associated with the lowest NTD risk (54). However, this was a large scale epidemiological-based study which did not include all pre-fortification NTD-associated factors in the analysis, such as alcohol use, and lacked detailed information regarding FA supplement use, such as dosage, which limits the practical application of the information.

The purpose of this study was to evaluate a comprehensive list of variables previously shown to be associated with NTDs or folate status, and include information on genotyping of the C677T polymorphism and on FA supplement use, to assess which variables were significantly associated with RBC concentrations ≥ 906 nmol/L. The long-term goal of this research is to develop a tool that could be used by health care professionals to provide advice to WCBY on their risk of suboptimal NTD-protective RBC folate concentrations and provide practical information to improve folate status.

5.3. SUBJECTS & METHODS

Participants

A convenience sample of one hundred and one healthy women (aged 18 to 45 y) were co-recruited with another study, from staff at the Hospital for Sick Children, individuals in the
greater Toronto area, and students from the University of Toronto, through advertisements and word of mouth, between April and December 2011. Women were excluded if they were: pregnant or lactating; consuming medications known to interfere with folate absorption; had a history of a disorder or condition that could interfere with the absorption, distribution, metabolism or excretion of folate; or, had a pre-existing condition that would increase the risk of pregnancy complicated by NTD as these women may have increased folate requirements (105). The study protocol was approved by the Research Ethics Board of the Hospital for Sick Children. Written informed consent from each participant was obtained on their study visit.

**Predictors**

This was a cross-sectional observational study examining the association of a comprehensive list of socio-demographic, dietary and lifestyle factors with RBC folate concentration above and below 906 nmol/L (51-54). Variables were derived from the factors found to be significant in the pre-fortification literature, those tested in the Canadian Health Measures Study and genetic information on the C677T methylenetetrahydrofolate reductase (MTHFR) genotype, as this has been shown to influence folate status. Further, we added additional questions to derive more detailed dietary intake and supplement usage information in order to capture particulars about the relationships between total folate intakes and more individual sources (e.g. types of fortified foods) as well as frequency and dose of FA-containing supplements.

Participants fasted overnight and refrained from taking any supplements the morning of the study visit in the Clinical Investigation Unit at The Hospital for Sick Children. During their visit, participants filled out a comprehensive socio-demographic, lifestyle, dietary and supplement use questionnaire and the U.S. adult validated Block Folic Acid/Dietary Folate
Equivalents Screener (266). The screener was not adjusted to reflect Canadian fortification for folic acid as the individual food values was not available.

Socio-demographic and lifestyle related data

Socio-demographic and lifestyle related data on age, income, education, ethnicity, country of birth (Canada or other), smoking, and alcohol use were obtained using the questionnaire. Income was categorized as <$25,000, $25,000-$64,999, and ≥$65,000. Education was categorized as education at or below an undergraduate degree or above an undergraduate degree. Self-reported alcohol use was categorized as don’t drink, < 1 drink/wk, or ≥ 1 drink/wk. Ethnicity was categorized as Caucasian, Asian, and other ethnicities. Smoking and born in Canada were categorized as yes or no. Some variables were collapsed to improve cell size and revised categories were modelled to closely represent those found in the literature.

Dietary folate intake data collection

Data on dietary folate intake were collected using both the Block screener to calculate total folate intake and separate questions focusing on significant sources of dietary folate and folic acid fortified foods, including: ready to eat cereals; fortified bread products; pastas, leafy greens; dark greens; vegetables; orange juice/oranges; and legumes. Questions were specified to capture frequency of consumption (e.g. number of servings per day/week/month/year) of each food group. The frequencies of consumption were then converted to a common measure of number of servings per day (274). For the purpose of analysis, questions on frequency of consumption were collapsed into two variables: fortified foods (white bread, pasta, and ready-to-eat breakfast cereals) and fruit, legumes and vegetables (orange juice and oranges, legumes, dark greens, leafy greens and vegetables).
Use of folic acid and B12 containing supplements

Information on each participant’s use of FA containing and/or B12 containing supplements in the past 30 d was captured in the administered questionnaire. Those who responded “yes” to the use of supplements were asked for details about supplement brand, dosage, and frequency of consumption for the consumed supplements. Information on use of fortified meal replacements, power bars or shakes was also collected. FA dosage reported by participants was verified with product labels and monographs. As the supplement forms of FA and B12 were consumed as part of a multivitamin/mineral formula, rather than as individual nutrients, the two questions asking subjects if they used FA containing supplements and B12 containing supplements (Y/N) supplements was re-categorized into a single variable about whether or not a multivitamin supplement was used (Y/N). More detailed information about how much FA was consumed in a supplement dose was calculated based on the amount of FA contained in the supplement used by how often the supplement was consumed to create FA dose (µg/d). Information about how often a FA containing supplement was consumed or the frequency of use was categorized as none, ≤1/wk, ≥2/wk.

Measurement of RBC folate concentrations

Venous blood samples were collected into vacutainers containing EDTA by a registered nurse. Whole blood samples were diluted 10-fold with (1% wt:vol) ascorbic acid to hemolyze RBC and then were incubated for 60 minutes at 37°C to allow conversion of folates to their microbiologically assay form. Hemolysates were then frozen at -80°C for later analysis of total RBC folate concentration (142). Remaining whole blood was separated (850 x g for 15 minutes at 4°C) by centrifuge into plasma and red blood cells. Theuffy coat was used for C677T
MTHFR genotyping (267). Sodium ascorbate (1%) was added to plasma samples to stabilize the folate and frozen for later folate content analysis.

Total whole blood hemolysate and plasma folate concentrations were measured by the microbiological assay as described by Molloy and Scott (142), using the chloramphenicol-resistant test organism Lactobacillus rhamnosus (ATCC27773, American Type Tissue Culture Collection, Manassas, VA) and 5-methyltetrahydrofolate from Merck Cie (Schaffhaussen, Switzerland) to generate the calibration curve. RBC folate content was calculated by difference, using the whole blood folate concentrations minus the plasma folate concentrations and corrected for hematocrit. A whole blood folate international standard (95/528, certified content of 22.1 nmol/L, National Institutes for Biological Standards and Control, Hertfordshire, United Kingdom) was used in every run to assess the accuracy and precision of the folate bioassay. The overall inter-assay coefficient of variation for the whole blood folate standard was 4.6% with a mean value of 21.5±2.8 nmol/L.

There is no internationally-recognized cut-off for sub-optimal maternal folate status associated with risk reduction of NTD. For the analyses presented herein we used the results of Daly et al (9) who reported a continuous dose-response relationship between RBC folate concentrations (up to 1292 nmol/L) and NTD risk to establish a proposed cut-off. The lower boundary of the uppermost RBC folate concentration presented in the Daly et al paper, measured by microbiologic assay (calibrated with folic acid) and the lowest risk of NTD birth was represented by a cut-off of 906 nmol/L. There is a certain degree of uncertainty in applying the results of the Daly et al (9) study to Canadian populations due to differences in NTD risk and FA fortification of the food supply, but at this time information regarding RBC folate concentrations associated with NTD protection for a Canadian population is limited. The current study also measured RBC folate concentrations using the microbiological assay; however the calibration
curve was calculated using 5-methyltetrahydrofolate instead of folic acid which requires an adjustment to compare data. While 5-methyltetrahydrofolate produces more accurate results, the results are approximately 25% lower than when FA is used as a calibrator so an adjustment to 738 nmol/L for the cutoff concentration was required for comparison (143).

Statistical analysis

A post hoc sample size calculation showed that our multiple linear regression modelling had 96% power with a sample size of 101 at a 5% significance level and 12 covariates, while our logistic regression modelling showed only moderate association using 6 covariates at 80% power (275).

Descriptive statistics for continuous variables were summarized as means ± SD while categorical variables were summarized as proportions for the sociodemographic, life-style, supplement and dietary characteristics of the participants. RBC folate concentration data were assessed for normal distribution and were log-transformed for use in the statistical analyses. No interactions were found between any of the tested variables and the RBC outcomes. The variance inflation factor (VIF) was estimated for all sociodemographic, lifestyle, diet and supplement variables used in the linear regression analyses. A VIF value greater than 10 was considered an indication of co-linearity and correlates were adjusted as follows. There was a high level of collinearity (r=0.768, P<0.0001) between FA and B_{12} supplement use. As all subjects who consumed vitamin B_{12} did so as part of a multivitamin supplement, a compiled variable called ‘multisupplement’ was created. Strong inter-correlations existed between ‘born in Canada’ and ‘ethnicity’ (r=0.313, P=0.0015) and between ‘income’ and ‘education’ (r=0.382, P<0.0001), so only ethnicity and education were used in the general linear models.
Correlation coefficients (r) were first used to examine associations between covariates and RBC folate concentrations (data not shown). Multiple linear regression analysis (continuous outcome) was used to test: 1) multisupplement use (y/n); 2) FA supplement dose (µg/d); and, 3) FA supplement frequency (none, ≤ 1/wk, ≥ 2/wk) along with the remaining sociodemographic and lifestyle variables (age, ethnicity, education, smoking, alcohol use) and dietary folate intakes (total folate intake [µg/L] or servings of folate-containing food groups, either FA fortified or plant food based) to determine factors that were significantly associated with RBC folate concentrations in three different models. All variables were initially entered into the model in order to determine the overall effect of the comprehensive list of sociodemographic, lifestyle and dietary factors in predicting RBC folate status. The final model was determined using backwards selection using a Wald chi-squared P-value < 0.1 to determine what variables should remain in the model.

Multiple logistic regression analyses were conducted to calculate odds ratios (OR) for factors associated with a folate status associated with NTD protection (≥ 738 nmol/L). Any covariate with a Wald chi-squared P-value < 0.1 from the continuous outcome multiple linear regression analysis was considered as a candidate variable for the dichotomous outcome logistic regression assessment. The Receiver Operating Characteristic (ROC) curve plotted the sensitivity and specificity of each model using a pre-determined cut-point of below (< 738 nmol/L) or above (≥ 738 nmol/L) NTD protective folate status, and compare the predictive powers of the logistic models. Area under ROC curve comparison statements were used to provide point and confidence interval estimates of each models area and provide pairwise differences between the models. In either FA supplement dose or FA supplement use alone to see if the model was significantly improved with the addition of the other variables. Area under ROC curve (AUC) was used to determine the quality of the tested models with 1 being a perfect
predictor and 0.5 being a model no better than chance. ROC contrast statements implement a non-parametric approach to statistically compare the AUC of ROC curves (276). An effect plot was also constructed based on the observed values showing the predicted probability of achieving a NTD protective folate status with increasing FA supplement doses. All statistical analysis was done using SAS software (version 9.3; SAS Institute Inc, Cary, NC). A P<0.05 was considered statistically significant in all analysis.

5.4. RESULTS

Descriptive

Means for continuous variables and RBC folate concentrations as well as comparisons by RBC folate concentrations < 738 nmol/L or ≥ 738 nmol/L by socio-demographic, lifestyle and genetic characteristics are shown in Table 5.1. The mean age of women in our sample was 28.7 ±7 years and all had completed some form of post-secondary education. The majority (60%) were students at the time of data collection. The overall mean RBC folate concentration of the sample was 849 ± 265 nmol/L and no woman had a RBC folate concentration below the cut-off for chronic folate deficiency (<305 nmol/L). Fifty-eight percent of women had RBC folate concentrations associated with NTD protection (≥ 738 nmol/L). There was no difference in RBC folate concentrations with income, birth status, smoking, or C677T MTHFR genotype but there were differences with ethnicity, alcohol intake, education, multisupplement use and frequency of FA supplement use (Table 1). Twenty-six women reported taking multivitamin supplements containing FA and B₁₂ and an additional 4 women consumed meal replacement bars and/or vitamin-containing drinks. Hence, in total 30 women (30%) consumed some form of supplemental FA in addition to dietary folate (naturally occurring or added as a fortificant). The mean daily dose of supplemental FA consumed by women was 68 ± 164 µg, but among
“supplement users” only, the average intake was 225 ± 234 µg or approximately 1 regular multivitamin taken every other day. There was no difference in age, income or dietary folate intake between supplement users and non-users (data not shown).

**Multiple Linear Regression**

The results of the different initial comprehensive models for both FA supplement dose (µg/d) and FA supplement frequency of use (none, ≤ 1/wk, ≥ 2/wk) are shown in Table 5.2. Whether or not a multisupplement was used was also tested with all the variables in the comprehensive model \((F=2.90, P= 0.0022, r^2 = 0.30, r^2 (adj) = 0.20)\) (data not shown) but the results were less predictive than either the FA supplement frequency of use (Model 1) or FA supplement dose (Model 2) models. Backwards selection, using 0.1 as a cutoff, was used to determine the final models for both Model 1 and Model 2. For the final FA supplement frequency of use model \((F=8.15, P< 0.0001, r^2 = 0.31, r^2 (adj) = 0.28)\), FA frequency of use; other ethnicity; <1 drink alcohol/week; >undergraduate degree were all individually associated with RBC folate concentrations (all \(P< 0.05\)) while T/T for C677T MTHFR was not significant \((P=0.0588)\) (data not shown). For the final FA supplement dose model \((F=8.37, P < 0.0001, r^2 = 0.32, r^2 (adj) = 0.28)\), FA supplement dose; other ethnicity; <1 drink alcohol/week; >undergraduate degree were all individually associated with RBC folate concentrations (all \(P< 0.04\)) while t/t for C677T MTHFR was not significant \((P=0.0943)\) (data not shown).

Sensitivity analysis was conducted to see if women who reported not taking a FA containing supplement (n=75) differed from the group as a whole (See Appendix Table 5.1). The final model showed that having an ethnic minority status \((0.0257)\) and consuming less than 1 drink of alcohol/wk \((p=0.027)\) were significant negative factors on folate status. Age \((p=0.0711)\) was not significant in this model \((F=5.32, P<0.0024, r^2 = 0.18, r^2 (adj) = 16)\).
Multiple Logistic Regression

Multiple logistic regression was used to determine the association between folate status associated with NTD protection (≥ 738 nmol/L, dichotomous outcome) and any covariates with a Wald chi-squared P-value < 0.1 from the continuous multiple regression above. Of the 30 women who reported taking multisupplements containing FA (not including the 4 women who took FA in meal replacement bars/vitamin drinks), 77% had RBC folate concentrations above the RBC folate cutoff associated with NTD protection (Table 5.1). Among the 58 women with RBC folate ≥ 738 nmol/L, 22 consumed some form of FA either as a supplement or meal replacement (Table 5.1). Logistic regression analysis results are shown in Table 5.3. To determine what FA supplement dose and what frequency of supplement use were associated with an NTD protective folate status both daily supplement FA dose (µg/d) and frequency of FA supplement use (none, ≤ 1/wk, ≥ 2/wk) were modeled. The scale of the predictor variable FA supplement dose was changed to reflect a 100µg/d increase of FA. In the final models (data not shown) for Model 2, each 100µg/d increase in supplemental FA dose improved folate status 1.9 times (95% confidence interval (CI): 1.2, 3.2) while alcohol intake of less than 1 drink/wk had a weakly significant inverse effect (P=0.0546) putting women at risk in the odds of having sub-optimal folate concentrations by 66%. Women using a FA supplement at least twice a week (Model 1) were more likely to have folate concentrations above 738 nmol/L (OR = 12.6, 95% CI: 2.3, 70.2) while an alcohol intake of < 1 drink/wk increased the risk by 64% (95% CI: 0.13, 0.99) on the odds of having a folate status below 738 nmol/L (P=0.0491). In the final models, controlling for education and ethnicity attenuated both models and improved both significance and the confidence intervals of the variables of interest.
**ROC analysis**

ROC analysis was then used to test the ability of the models to predict how likely women were to achieve a folate status associated with NTD protection and compare the possible models to the predictive ability of FA supplementation alone. Table 5.4 shows the contrasts of AUC for the various predictive functions of the final models. The results of Model 1 suggest that the effect of alcohol intake and controlling for ethnicity significantly improves the ability to predict the likelihood of achieving an NTD protective folate status (AUC = 0.73, P = 0.0212) compared to accounting for FA supplement frequency of use alone (AUC = 0.61). Model 2 is similar in that considering ethnicity and alcohol intake with FA supplement dose improves the ability to predict the likelihood of achieving an NTD protective folate status (AUC = 0.73, P = 0.0143) compared to FA supplement dose alone (AUC = 0.61) or when accounting for education with low alcohol intake (AUC = 0.70, P = 0.0709). The AUC of both final models were above the probability suggested by chance indicating that either model is capable of identifying a RBC folate concentration associated with NTD protection. Appendix Figure 5.1 shows the predictive probability of the odds of having an NTD protective status at increasing FA supplement doses. A FA supplement intake of 200 µg/d (or a multisupplement taken every other day) has a predictive probability of ~ 75% of having a RBC folate concentration ≥ 738 nmol/L.

5.5. **DISCUSSION**

Increased folate status during reproduction is necessary not only to reduce the risk of NTD but also to support the rapid growth of fetal and maternal tissue (67, 92). Since FA fortification of the food supply, less than 1% of the North American population are now overtly
folate deficient (<305 nmol/L); however 22% of WCBY have RBC folate concentrations below that lower range cut-off associated with NTD protection (≥906 nmol/L) (16). Conversely, many women in North America now have supra-physiological levels of circulating folate and there is an emerging, albeit controversial, body of evidence suggesting adverse health effects to both mother and offspring at these high levels (16, 33, 37, 273). At the present time, it remains prudent to recommend women capable of becoming pregnant to consume 400 ug of synthetic FA during the periconceptional period to prevent NTDs. However, health care professionals concerned about the folate status of a patient may recommend higher doses due to poor adherence in consuming supplements or other factors in the literature typically ascribed to low folate status or increased risk of NTD. The obvious solution to this dilemma would be to measure RBC folate concentrations but this approach is expensive on a population level and many laboratories have either discouraged or restricted the use of RBC folate tests given the low prevalence of overt folate deficiency (15, 277). Hence, alternative methods such as questionnaires to assess the risk of having RBC folate concentrations less than maximally protective against NTDs would be invaluable. (50). The results of this study suggest that while higher FA supplement doses and use of FA supplements at ≥ 2/wk, ethnicity, education, and alcohol intake are associated with overall folate status either positively or negatively (Table 5.2), only regular doses of FA, at least twice a week, as well as asking information regarding alcohol intake in conjunction with ethnicity can provide an adequately predictive model for folate status ≥ 906 nmol/L (Table 5.4).

FA supplementation influences RBC folate status and is a strong contributor to the odds of having a folate status ≥ 906 nmol/L. Colapinto et al. (54) found that FA-containing multivitamin use is associated with a greater prevalence of RBC folate concentrations ≥ 906 nmol/L than in nonusers (87.9% vs 75.2%) but did not define either FA dose or frequency of FA supplement use. Pietrzik et al. (166) demonstrated that a FA dose of 400µg/d achieved RBC
folate concentrations of ~ 1000 nmol/L after 8 weeks and ~ 1300 nmol/L after 24 weeks in women, while higher doses of FA (1.1 and 5 mg) showed faster rates of accumulation and higher concentrations in a similar time frame (265). Conversely, lower daily doses of FA (140 µg/d) will also decrease the prevalence of women with concentrations < 906 nmol/L 2-fold while 400 µg/d FA will decrease it 3-fold with no difference in the odds after 40 weeks (167). These results are in line with our results showing that WCBY taking a multivitamin containing 400 µg ≥ 2/wk were 12.6 fold more likely to have RBC folate concentrations ≥ 906 nmol/L compared to those not using FA containing supplements. This multivitamin dose had a predicted probability of approximately 75% of a protective folate status with a FA supplement dose of 200 µg/d (or a multivitamin taken every other day).

Our analysis showed the complex nature of alcohol on folate status; we show a J-shape pattern in RBC folate concentrations with the highest risk of low concentrations in those with an alcohol intake of < 1 drink/wk. It is well established that high alcohol intake has a detrimental effect on normal folate metabolism by interfering with absorption, uptake and storage of folate, increasing urinary folate excretion, and inhibiting methionine synthase activity (193). However, evidence suggests detrimental health effects exist with low alcohol intakes, while modest alcohol intake can be protective against all-cause mortality and improve homocysteine, serum, and RBC folate concentrations, although results are inconsistent (187, 190, 196, 197, 199, 278). In the Nurse’s Health Study (278) the lowest rates of mortality for cancer and heart disease were found in those with an alcohol intake of 1-2 drinks/d while low folate intake (< 180 µg/d) enhanced the risk. Lower homocysteine and higher serum folate concentrations are associated with modest alcohol intakes versus low intakes of < 30 drinks/month (197). In comparison, a significant inverse linear relationship in both serum and RBC folate concentrations with increasing alcohol intake was seen in U.S. data (187). Laufer et al. (196) found no difference in
serum folate concentrations in post-menopausal women consuming 0, 1 drink/d, and 2 drink/d of alcohol for 8 weeks in a cross-over design study; however, these women consumed 100% of the DRI for all vitamins and minerals for the study duration. None of the above studies differentiated the type of alcohol consumed. The Danish Inter99 Study (190) looked at total alcohol intake as well as intake from beer and wine. There was a positive dose response with serum folate concentrations and total alcohol and beer intake but a significant J-shape relationship with increasing wine intake suggesting that the type of alcoholic beverage consumed may have an effect (190). Beer does contain B vitamins and modest intakes would positively contribute to folate status (199) while those who do not consume beer would not benefit from this folate source. In this study we did not delineate between alcohol types which may explain why we did not see any significance at higher alcohol intakes.

The lack of any relationship with enriched white bread products was surprising since fortification increases intake \( \sim 100 \mu g/d \) (12). Mason (174) suggests that while habitual intake is a major determinant of nutritional status, overall biostatus is influenced by a number of factors (bioavailability, genetics etc) of which dietary intake is only one small aspect. Houghton et al. (41) demonstrated that dietary folate intake is not predictive of blood folate concentrations when women are exposed to higher folate intakes. Evidence suggests that an increased intake of refined grain products is associated with lower overall folate intake because the overall diet quality is lower (181, 279). Higher scores on the healthy eating index (HEI) are associated with higher intakes of fruits, vegetables and whole grains and significantly less risk of inadequate nutrient intakes (181, 279, 280). We found a weak inverse correlation between the intakes of fortified foods and fruits, legumes and vegetables \((r= -0.17, P=0.0860)\). We were unable to determine an income effect in our population, unlike previous data (54) due to the high proportion of students and overall high level of education. However, national data suggests that higher income and
education are associated with more healthful dietary patterns including higher intakes of fruits, vegetables and whole grains (280-282) and higher use of supplements (40, 54, 163). We did find that women consuming more servings fruits, legumes and vegetables were more likely to use supplements (r=0.31, P=0.0021).

This study was limited by the sample size of our convenience sample. While 101 women was sufficient to identify factors associated with folate status in multiple regressions, the reduced power in the binomial regression suggest the study may have been too small to adequately explore risks associated with higher intakes of alcohol, the influence of diet, or ethnicity. For example, studies using national data have shown that ethnicity is related to folate status (187). We had three participants of Latin American background with significantly lower RBC folate concentrations (607.6 ± 22.4 nmol/L, P<0.05) but they were grouped with other ethnicities (with higher RBC folate concentrations) for purposes of analysis which limited our ability to tease out the influence of ethnicity. Use of a convenience sample might reduce the generalizability of our results since a high proportion of participants were students with or seeking postsecondary degrees.

In conclusion, an extensive list of factors is not required to assist clinicians in predicting RBC folate status in WBCY who are planning in becoming pregnant. These results suggest that ensuring WBCA are taking a FA containing supplement at least twice a week and assessing alcohol use, as low intake may require WCBY to use supplements more often, will assist clinicians in adequately determining the probability of a woman having optimal folate status to reduce the risk of folate-related NTD.

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<table>
<thead>
<tr>
<th>Folate status</th>
<th>RBC folate concentration&lt;738 nmol/L (n=43)</th>
<th>RBC folate ≥738 nmol/L (n=58)</th>
<th>P^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC folate concentration (nmol/L)</td>
<td>849 ± 2653</td>
<td>607 ± 94</td>
<td>1030 ± 197</td>
</tr>
<tr>
<td>Age (y)</td>
<td>28.7 ± 7</td>
<td>29.3 ± 7.5</td>
<td>28.2 ± 6.7</td>
</tr>
<tr>
<td>Dietary folate intake (µg)</td>
<td>388 ± 136</td>
<td>386 ± 132</td>
<td>389 ± 141</td>
</tr>
<tr>
<td>FA fortified foods (serv/d)</td>
<td>1.31 ± 0.94</td>
<td>1.42 ± 1.0</td>
<td>1.22 ± 0.9</td>
</tr>
<tr>
<td>Fruits, legumes &amp; vegetable intake (serv/d)</td>
<td>3.25 ± 1.72</td>
<td>3 ± 3</td>
<td>3.5 ± 2.7</td>
</tr>
<tr>
<td>Supplement FA/d^4</td>
<td>225 ± 234</td>
<td>93 ± 93</td>
<td>273 ± 252</td>
</tr>
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<td>Multisupplement use [n, (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>975 ± 285*</td>
<td>6(14)</td>
<td>20(34)</td>
</tr>
<tr>
<td>no</td>
<td>806 ± 244</td>
<td>37(86)</td>
<td>38(66)</td>
</tr>
<tr>
<td>FA supplement frequency [n, (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>797 ± 244</td>
<td>35 (81)</td>
<td>36 (62)</td>
</tr>
<tr>
<td>modest use (≤ 1/wk)</td>
<td>842 ± 231*</td>
<td>7(16)</td>
<td>8(14)</td>
</tr>
<tr>
<td>regular use (≥ 2/wk)</td>
<td>1109 ± 252*</td>
<td>1(2)</td>
<td>14 (24)</td>
</tr>
<tr>
<td>Ethnicity [n, (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caucasian</td>
<td>881 ±269</td>
<td>23(55)</td>
<td>32(57)</td>
</tr>
<tr>
<td>asian</td>
<td>869 ±242</td>
<td>7(17)</td>
<td>15(27)</td>
</tr>
<tr>
<td>other ethnicities</td>
<td>730 ± 254*</td>
<td>12(29)</td>
<td>9(16)</td>
</tr>
<tr>
<td>Alcohol use [n, (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>don't drink</td>
<td>907 ± 252</td>
<td>6(14)</td>
<td>15(26)</td>
</tr>
<tr>
<td>&lt;1 drink/week</td>
<td>751 ± 253*</td>
<td>18(42)</td>
<td>14(25)</td>
</tr>
<tr>
<td>≥1 drink/week</td>
<td>893 ± 266</td>
<td>19(44)</td>
<td>28(49)</td>
</tr>
<tr>
<td>Education [n, (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤undergraduate degree</td>
<td>905 ± 281</td>
<td>21 (49)</td>
<td>33 (60)</td>
</tr>
<tr>
<td>&gt;undergraduate degree</td>
<td>767 ± 216*</td>
<td>22 (51)</td>
<td>22 (40)</td>
</tr>
<tr>
<td>Genotype C677T MTHFR [n, (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wild type</td>
<td>852 ± 304</td>
<td>23 (53)</td>
<td>27 (47)</td>
</tr>
<tr>
<td>heterozygous</td>
<td>868 ± 217</td>
<td>12 (28)</td>
<td>24 (41)</td>
</tr>
<tr>
<td>homozygous</td>
<td>796 ± 234</td>
<td>8 (19)</td>
<td>7 ( 12)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>741 ± 124</td>
<td>3 (7)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>No</td>
<td>857 ± 270</td>
<td>40 (93)</td>
<td>55 (96)</td>
</tr>
</tbody>
</table>
### Income

<table>
<thead>
<tr>
<th>Income</th>
<th>Count</th>
<th>Mean ± SD</th>
<th>Min-Max</th>
<th>% of Total</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 25 000</td>
<td>41</td>
<td>877 ± 289</td>
<td>18 (42)</td>
<td>23 (41)</td>
<td>0.6479</td>
</tr>
<tr>
<td>25 000-64 999</td>
<td>43</td>
<td>836 ± 258</td>
<td>17 (40)</td>
<td>26 (46)</td>
<td></td>
</tr>
<tr>
<td>&gt;65 000</td>
<td>15</td>
<td>807 ± 233</td>
<td>8 (19)</td>
<td>7 (13)</td>
<td></td>
</tr>
</tbody>
</table>

### Born in Canada

<table>
<thead>
<tr>
<th>Born in Canada</th>
<th>Count</th>
<th>Mean ± SD</th>
<th>Min-Max</th>
<th>% of Total</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>64</td>
<td>878 ± 282</td>
<td>26 (60)</td>
<td>38 (66)</td>
<td>0.6779</td>
</tr>
<tr>
<td>No</td>
<td>37</td>
<td>801 ± 228</td>
<td>17 (40)</td>
<td>20 (34)</td>
<td></td>
</tr>
</tbody>
</table>

1. Folate status defined as < or ≥ 906 nmol/L (equivalent to 738 nmol/L when microbiological assay calibrated with 5-methyltetrahydrofolate) (n=101); FA, folic acid; serv/d, servings per day. FA, folic acid; MTHFR, methylenetetrahydrofolate reductase.
2. Mean ± SD (all such values).
3. Significance reported for Satterthwaite F statistic (p<0.05) for values reported as means ± SD; Significance reported for Chi-square and Fisher’s exact test performed for values reported as n (%).
4. Folic acid supplement dose includes those taking either FA containing supplements and/or meal replacements & vitamin drinks.
* indicates a significant difference with P< 0.05 for group comparison.
Table 5.2
Multiple regression analyses of the association of sociodemographic, lifestyle and dietary factors with red blood cell folate concentrations

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta )</td>
<td>SEE</td>
<td>( \text{pr}&gt;[t] )</td>
<td>( \beta )</td>
<td>SEE</td>
<td>( \text{pr}&gt;[t] )</td>
</tr>
<tr>
<td>Intercept</td>
<td>6.805</td>
<td>0.203</td>
<td>&lt;0.0001</td>
<td>6.890</td>
<td>0.200</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FA supplement frequency</td>
<td>0.327</td>
<td>0.093</td>
<td>0.0007</td>
<td>0.001</td>
<td>0.000</td>
<td>0.0008</td>
</tr>
<tr>
<td>( \geq 2 ) / wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplement dose FA/d (µg/d)</td>
<td>-0.001</td>
<td>0.005</td>
<td>0.8177</td>
<td>-0.002</td>
<td>0.005</td>
<td>0.6888</td>
</tr>
<tr>
<td>Age</td>
<td>-0.012</td>
<td>0.087</td>
<td>0.8874</td>
<td>-0.008</td>
<td>0.874</td>
<td>0.9240</td>
</tr>
<tr>
<td>Other ethnicities</td>
<td>-0.191</td>
<td>0.082</td>
<td>0.0218</td>
<td>-0.168</td>
<td>0.081</td>
<td>0.0418</td>
</tr>
<tr>
<td>&lt; 1 drink/wk</td>
<td>-0.150</td>
<td>0.087</td>
<td>0.0903</td>
<td>-0.185</td>
<td>0.858</td>
<td>0.0339</td>
</tr>
<tr>
<td>( \geq 1 ) drink/wk</td>
<td>-0.019</td>
<td>0.090</td>
<td>0.6317</td>
<td>-0.041</td>
<td>0.089</td>
<td>0.6489</td>
</tr>
<tr>
<td>Undergraduate degree</td>
<td>-0.131</td>
<td>0.067</td>
<td>0.0525</td>
<td>-0.130</td>
<td>0.067</td>
<td>0.0541</td>
</tr>
<tr>
<td>Smoke</td>
<td>-0.165</td>
<td>0.137</td>
<td>0.2324</td>
<td>-0.133</td>
<td>0.137</td>
<td>0.3325</td>
</tr>
<tr>
<td>Fortified foods</td>
<td>-0.009</td>
<td>0.036</td>
<td>0.8121</td>
<td>-0.016</td>
<td>0.035</td>
<td>0.6589</td>
</tr>
<tr>
<td>Fruit, legumes &amp; vegetables (^4)</td>
<td>0.018</td>
<td>0.012</td>
<td>0.3197</td>
<td>0.007</td>
<td>0.019</td>
<td>0.7222</td>
</tr>
<tr>
<td>C677T MTHFR genotype (c/t)</td>
<td>0.015</td>
<td>0.072</td>
<td>0.8301</td>
<td>0.017</td>
<td>0.072</td>
<td>0.8125</td>
</tr>
<tr>
<td>C677T MTHFR genotype (t/t)</td>
<td>-0.133</td>
<td>0.089</td>
<td>0.1401</td>
<td>-0.148</td>
<td>0.890</td>
<td>0.1001</td>
</tr>
</tbody>
</table>

\( Model 1: F=3.09, P=0.0010, r^2 = 0.34, r^2(\text{adj})= 0.23 \)

\( Model 2: F=3.29, P=0.0007, r^2 = 0.33, r^2(\text{adj})= 0.23 \)

---

\(^1\) General linear model analyses were used (n=101) for all models. FA, folic acid; MTHFR, methylenetetrahydrofolate reductase

\(^2\) Comprehensive model using FA supplement frequency of use equivalent to 2 or more supplements containing 400 mcg FA/wk

\(^3\) Comprehensive model using FA supplement dose per day (µg/d)
TABLE 5.3

Folic acid supplement information, ethnicity, alcohol use, education and folate concentrations associated with NTD protection in women of childbearing years

<table>
<thead>
<tr>
<th>Factors</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD</td>
<td>OR (95 % CI)</td>
</tr>
<tr>
<td>FA supplement frequency none</td>
<td>1</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>FA supplement frequency ≥2/wk</td>
<td>1</td>
<td>13.59 (1.94, 95.13)</td>
</tr>
<tr>
<td>Supplement dose FA/d (µg/d)²</td>
<td>1</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>1</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>Asian</td>
<td>1</td>
<td>1.78 (0.60, 5.30)</td>
</tr>
<tr>
<td>Other ethnicities</td>
<td>1</td>
<td>0.47 (0.16, 1.40)</td>
</tr>
<tr>
<td>Don’t drink</td>
<td>1</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>&lt;1 drink/week</td>
<td>1</td>
<td>0.35 (0.11, 1.17)</td>
</tr>
<tr>
<td>≥ 1 drink/week</td>
<td>1</td>
<td>0.75 (0.22, 2.55)</td>
</tr>
<tr>
<td>&lt;Undergraduate degree</td>
<td>1</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>&gt; Undergraduate degree</td>
<td>1</td>
<td>0.9 (0.39, 2.0)</td>
</tr>
</tbody>
</table>

¹ Multiple logistic regression analyses were used (n=101) for all models; folate concentrations associated with NTD protection defined as < or ≥ 906 nmol/L (equivalent to 738 nmol/L when microbiological assay calibrated with 5-methyltetrahydrofolate). FA, folic acid; serv/d, servings per day; MTHFR, methylenetetrahydrofolate reductase.

² Final model uses FA supplement frequency of use defined as no supplement, modest and regular supplement use, controlling for education; significance is at the 0.10 level.

³ Model tests predictors used in general linear model, supplement dose (µg/d) includes intakes from all study participants; significance is at 0.1 level.

⁴ The supplement FA/day is based on supplement dose X frequency of use. The SD is set to reflect unit increases by 100 µg.
### TABLE 5.4
Comparisons of area under the Receiving Operating Characteristic curve for predictive models of having folate concentrations associated with NTD protection

<table>
<thead>
<tr>
<th>Model</th>
<th>Independent variable</th>
<th>AUC (95% CI)</th>
<th>P value for comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FA supplement frequency of use ≥2/wk alone</td>
<td>0.61 (0.53, 0.70)</td>
<td>[0.5872]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + education</td>
<td>0.63 (0.52, 0.73)</td>
<td>[0.5872]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + alcohol intake</td>
<td>0.68 (0.57, 0.78)</td>
<td>[0.1518]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + ethnicity</td>
<td>0.69 (0.59, 0.79)</td>
<td>[0.1007]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + education + ethnicity</td>
<td>0.70 (0.59, 0.80)</td>
<td>[0.0765]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + alcohol intake + education</td>
<td>0.68 (0.58, 0.79)</td>
<td>[0.2305]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + alcohol intake + ethnicity</td>
<td>0.72 (0.62, 0.82)</td>
<td>[0.0212]</td>
</tr>
<tr>
<td></td>
<td>FA frequency of use ≥ 2/wk + alcohol intake + education + ethnicity</td>
<td>0.73 (0.63, 0.83)</td>
<td>[0.0188]</td>
</tr>
<tr>
<td>2</td>
<td>FA supplement dose (µg/d) alone</td>
<td>0.61 (0.52, 0.69)</td>
<td>[0.5529]</td>
</tr>
<tr>
<td></td>
<td>FA supplement dose (µg/d) + education</td>
<td>0.63 (0.52, 0.73)</td>
<td>[0.0965]</td>
</tr>
<tr>
<td></td>
<td>FA supplement dose(µg/d) + alcohol intake</td>
<td>0.68 (0.58, 0.79)</td>
<td>[0.0994]</td>
</tr>
<tr>
<td></td>
<td>FA supplement dose (µg/d) + ethnicity</td>
<td>0.67 (0.57, 0.78)</td>
<td>[0.115]</td>
</tr>
<tr>
<td></td>
<td>FA supplement dose(µg/d) + alcohol intake + education</td>
<td>0.68 (0.57, 0.79)</td>
<td>[0.0709]</td>
</tr>
<tr>
<td></td>
<td>FA supplement dose (µg/d) + alcohol intake + education + ethnicity</td>
<td>0.70 (0.59, 0.80)</td>
<td>[0.0143]</td>
</tr>
<tr>
<td></td>
<td>FA supplement dose (µg/d) + alcohol intake + education + ethnicity</td>
<td>0.73 (0.62, 0.82)</td>
<td>[0.0137]</td>
</tr>
</tbody>
</table>

1 ROC contrast estimation and testing were used (n=101) for all models. Concentrations associated with NTD protection defined as 906 nmol/L (equivalent to 738 nmol/L when microbiological assay calibrated with 5-methyltetrahydrofolate). AUC, area under the curve; FA, folic acid; NTD, neural tube defect.

2 P values were calculated for Chi-Square statistic. Model 1: subsequent models were compared to AUC of FA supplement frequency of use alone Model 2: subsequent models were compared to AUC of FA supplement dose alone;
Appendix Table

Appendix Table 5.1

Multiple regression analyses of the association of sociodemographic, lifestyle and dietary factors with red blood cell folate concentrations in women of childbearing years not taking FA supplements

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>SEE</td>
</tr>
<tr>
<td>intercept</td>
<td>6.990</td>
<td>0.240</td>
</tr>
<tr>
<td>age</td>
<td>-0.006</td>
<td>0.005</td>
</tr>
<tr>
<td>Asian</td>
<td>0.020</td>
<td>0.100</td>
</tr>
<tr>
<td>other ethnicities</td>
<td>-0.190</td>
<td>0.102</td>
</tr>
<tr>
<td>&lt; 1 drink/wk</td>
<td>-0.230</td>
<td>0.103</td>
</tr>
<tr>
<td>≥ 1 drink/wk</td>
<td>-0.090</td>
<td>0.106</td>
</tr>
<tr>
<td>&gt; undergraduate degree</td>
<td>-0.110</td>
<td>0.081</td>
</tr>
<tr>
<td>smoke</td>
<td>-0.170</td>
<td>0.160</td>
</tr>
<tr>
<td>fortified foods</td>
<td>0.006</td>
<td>0.040</td>
</tr>
<tr>
<td>fruit, legumes &amp; vegetables³</td>
<td>-0.005</td>
<td>0.030</td>
</tr>
<tr>
<td>C677T MTHFR genotype (c/t)</td>
<td>0.100</td>
<td>0.080</td>
</tr>
<tr>
<td>C677T MTHFR genotype (t/t)</td>
<td>-0.080</td>
<td>0.116</td>
</tr>
</tbody>
</table>

Model 1: F=1.91, p=0.0583, r²=0.27, r²(adj)=0.13

Model 2: F=5.32 p=0.0024, r²=0.20, r²(adj)=0.16

1 general linear model analyses were used (n=75) for all models; FA, folic acid; MTHFR, methylenetetrahydrofolate reductase.

2 Model generated using backwards selection entry using 0.1 as cut-off criteria.

³ Fruit, legumes, and vegetable intake includes: orange juice and oranges, dark greens, legumes, lettuce and vegetables.
Appendix Figure

Appendix Figure 5.1
Effect plot diagram showing the predicted probability (solid line) and the confidence limits (gray area) based on observed values (o) of achieving a folate status associated with neural tube defect protection with increasing folic acid supplement doses (µg/d).
6. THESIS STUDY # 3

Dietary sources of folate, vitamins B₆ and B₁₂, and choline among Canadian women of childbearing age: Do intakes of women of childbearing age approach adequate intake levels for choline?

6.1. ABSTRACT

Background: Evidence suggests that dysregulation of one carbon (1-C) metabolism can have adverse pregnancy and fetal health outcomes; therefore, optimal intake of nutrients involved in 1-C metabolism, specifically folate, vitamins B₆ and B₁₂ and choline, is essential. Key food sources for 1-C vitamins have never been systematically investigated at the national level in Canadian women of childbearing years (WCBY). Currently, there is no information about choline intakes in the Canadian population for any age group. Choline has recently been included in the Canadian Nutrient File (2010 version) (CNF) allowing us to adapt the information for use with the Canadian Community Health Survey (CCHS) 2.2. The objectives of this study were to: 1) derive the percent contribution of food sources for each 1-C nutrient (folate, B₆, B₁₂ and choline) for WCBY; and, 2) calculate the choline intakes for the Canadian population by both life stage and gender and estimate the prevalence of intakes below the adequate intake for each group.

Methods: Dietary recall data for folate, B₆ and B₁₂ were collected for all non-pregnant, non-lactating women between 18-45 y (n=4308) using CCHS 2.2 data. Nutrient information for choline was imported from the CNF and matched to all related foods in the CCHS 2.2 dietary recall data. Percent contributions and choline intakes were then estimated for all food sources in the survey for both WCBY and for the Canadian population ≥ 1y (n=34 840). Distributions of usual intakes of total choline for age and sex groups were estimated using PC SIDE (version 1.11).

Results: For WCBY, FA-fortified foods account for 45% of folate intake while fruits, vegetables and legumes contribute about 29%. Poultry, fluid milk, beef, potatoes and bananas were the top 5 contributors to B₆ intake accounting for 35% of intake. Milk, beef and cheese accounted for 50% of total B₁₂ intake. Dairy and eggs were the top food sources for choline (29%) with eggs contributing almost 12% alone, while fruit, vegetables and legumes were the next highest
contributors at 27%. The mean daily intake was 238 mg for choline with ~99% of WCBY having intakes below the AI.

Conclusions: Food sources for one carbon nutrients are varied in origin, but increased consumption of choline containing foods is needed.

6.2. INTRODUCTION

There is growing evidence that adequate intakes of all one-carbon (1-C) vitamins are essential for reproductive health and that dysregulation in any part of the pathway can lead to long term adverse health outcomes in offspring. While the evidence linking folate to neural tube defects (NTD) is strong, there is a growing body of evidence linking deficiencies of other 1-C vitamins such as B₆, B₁₂ and choline with various congenital deformities including craniosynostosis, cleft lip, heart defects, and NTD (243-245, 247-249, 253-256). Conversely, there is also evidence suggesting that high intakes of vitamin B₆ (pyridoxal 5-phosphate), vitamin B₁₂ or choline may have no effect or even increase the risk for such defects as metopic synostosis and cleft lip palate (244, 246, 252, 258). High intakes of folic acid (FA) may cause perturbations within 1-C metabolism such as exacerbating the signs of B₁₂ deficiency, altering DNA methylation which could impair hepatic lipid metabolism and influence cancer development (20, 106, 126, 283, 284). It has been suggested that the interrelationship between these 1-C nutrients can have a greater impact on methylation or on nucleotide biosynthesis than deficiency or high intakes of a single one carbon nutrient (57).

Given how critical the role of 1-C nutrients is in promoting healthy reproductive outcomes as well as long-term maternal health, adequate intakes of the 1-C vitamins are crucial for women of childbearing years (WCBY). Evidence suggests that approximately 27-32% of WCBY are using FA containing supplements either alone or in a multivitamin (55, 56). This will increase to approximately 55% periconceptionally and up to 84% in the first trimester with most consuming a multi- or prenatal- vitamin which will have the benefit of containing vitamins B₆ and B₁₂ but not choline (40, 170). Currently, choline is not included in the majority of multivitamin supplements including prenatal brands (226). The FA fortification of white wheat flour and select grains products labelled enriched in North America has been demonstrated to be successful in reducing NTD rates up to 46% (6, 14). In the U.S, ready-to-eat breakfast cereals
are heavily fortified up to 100% of the daily value and include vitamins B₆ and B₁₂; conversely, voluntary fortification of breakfast cereal in Canada mandates much lower limits for select nutrients and does not allow for the addition of B₁₂ (285). Currently, B₁₂ is only used to fortify select plant-based beverages and simulated meat and poultry products (285). The dietary intakes of folate, B₆ and B₁₂ are generally above requirements for WCBY (without the inclusion of supplements) in North America; however, fewer than 23% of U.S. women and up to 25% of Canadian women < 19 yrs have intakes below the EAR for folate, just over 12% of U.S. women and 13% of Canadian women have intakes below the EAR for B₆, while only 8% of U.S women and up to 12% Canadian women have inadequate B₁₂ intakes (59, 60, 179). Only a few small studies have looked at choline intake in WCBY and found that the majority have intakes below the adequate intake (AI) for choline (229, 286). However, dietary restrictions, either voluntary or involuntary could potentially affect the diet quality and potentially affect micronutrient intake (64). Thus an understanding of what food sources WCBY are consuming to meet their dietary requirement of these nutrients is crucial to address issues of diet quality, either with fortification or education, within the target group.

Choline is essential not only during the neonatal period to reduce NTD risk and for brain and memory development in the fetus but in wide-ranging roles including methyl metabolism, neurotransmitter synthesis, cell-membrane signalling, lipid transport and hepatic health both in fetal development and in adults (217). It is only recently that information on choline intakes has been collected on the population level starting with the 2005-2006 NHANES survey (63). At this time, there are no data on choline intakes in the Canadian population.

Currently, there is little data on food sources for folate, vitamins B₆, B₁₂ and choline for WCBY. Further, no national data on choline intakes have been determined for Canadian WCBY nor for the Canadian population as a whole. The Canadian Community Health Survey, Cycle 2.2 (CCHS 2.2) was the first nationally-represented dietary intake data collected in Canada in over 30 years and allows us the opportunity to look at foods chosen by Canadian women of reproductive age to meet their dietary intake for one carbon nutrients (287). Further, the recent inclusion of choline information to the Canadian Nutrient File (CNF [2010 version]) gives us the opportunity to calculate choline intakes and food sources for the Canadian population. The objectives of this study were to: 1) derive the percent contribution of top food sources for each one carbon nutrient (folate, B₆, B₁₂ and choline); and, 2) calculate the choline intake for the
Canadian population by both life stage and gender and estimate the prevalence of intakes below the AI for each group.

6.3. METHODS

Data Source and Participants
Data were taken from the CCHS 2.2 cycle, a nationally representative, cross-sectional survey, conducted between January 2004 and January 2005 with 35,107 respondents (287). The survey was conducted across 10 provinces and excluded individuals: living in the three Territories, on Indian Reserves and Crown lands, institutions, and full-time members of the Canadian Armed Forces (287). We applied to Statistics Canada in order for us to work with the original dataset. For WCBY, the sample was determined as follows: we included only women of reproductive age, defined as all women between the ages of 18 to 45 y (n=4,575) and then excluded any pregnant and lactating women (n=267) to reach the sample size of n=4,308. Given the lack of data on choline intakes among Canadians in general, when determining the mean daily intake for WCBY and the prevalence of intakes below the AI, we also looked at the intakes in the entire Canadian population. The Canadian population sample (n=34,840) excluded pregnant and lactating women (n=267), children < 1 y age, and those who only consumed breast milk (n=193). We stratified the Canadian population by both life stage and gender groups as defined in the Dietary References by the Institute of Medicine (288).

Dietary Intake Data Collection
Dietary intake data were based on a 24-h recall which used the Automated Multiple-Pass Method (287), a computer assisted interviewing methodology. A second 24-h recall was conducted, 3 to 10 days after the initial recall, with approximately 30% of respondents. For children under 5, parents answered the recall questions alone; for children between the ages of 6 and 11, both the child and the parent were allowed to answer while those ≥ 12 y completed the dietary interview themselves (287). The composition of foods for folate, vitamin B₁₂ and vitamin B₆ was derived from the CNF (supplemented 2001b version) and is itself is derived from the USDA Nutrient Database for Standard Reference and modified to reflect Canadian
fortification regulations (287). Values for nutrients in question were verified to ensure that fortification levels did reflect Canadian regulations and any discrepancies were corrected (see below for detail). To determine the choline content of foods the most recent version of the CNF (2010 version) (289), was imported and matched to corresponding foods codes via bridge files obtained from the Bureau of Nutrition Sciences at Health Canada. In CCHS 2.2, foods are coded using a unique food code for each food listed with a description and nutrient information per gram of food. It was built using information from several databases: the CNF; a recipe database of nearly 3000 recipes adapted from the USDA recipe database; and, a list of survey (new or local) foods (287). Foods are further grouped into a food group system (e.g. beef), classifying both basic foods and recipes, thereby allowing researchers to categorize and then summarize the dietary information collected in the survey (287).

**Nutrients Included in Analysis**

Fortification levels were checked for folate, $B_6$ and $B_{12}$ to ensure accuracy with Canadian regulations. The only discrepancy found was for FA concentrations in rice. This was corrected to reflect Canadian fortification regulations. Folate intake was calculated as dietary folate equivalents (DFE) and is based on the following calculation: $\text{DFE} = \mu g \text{ food folate} + (\mu g \text{ food folic acid [in fortified food products]} \times 1.7)$ (42). Choline was not included in the original CCHS 2.2 survey analysis; therefore, to calculate choline intakes, the data for choline was inputted from the most recent version of the CNF (2010 version) as described above and the information was used to calculate both the amount of choline in each portion of food consumed (amount choline/g X food amount in g) and the total the amount of choline consumed per day for each day of 24-h recall. The choline values in the 2010 version of the CNF are derived from the USDA Database for the Choline Content of Common Foods Release 2 (634 foods) (290) and that are used in the USDA National Nutrient Database for Standard Reference (233).

**Statistical Analysis**

All analysis was performed with SAS software (version 9.3; SAS Institute Inc, Cary, N.C.). Survey weights provided by Statistics Canada were used to account for all non-responses, the unequal probability of selection and the multistage sampling design (287). The weight variable corresponds to the number of people in the entire population that are represented by the surveyed
individual (287). For calculating of percent contribution of food sources, SAS SURVEY commands were used to account for the complexity of the survey design. Only the first day of the 24-h recall was used to calculate the percent contribution of the top food sources and daily mean intake. Food sources frequencies were generated by using a method originally reported by Block et al (291) to calculate the percent contribution of each food to the population’s total consumption of the nutrient in question by using the weighted sum of said nutrient in the amounts of all foods reported. The percent contribution is calculated as follows:

$$\frac{\sum_{j=1}^{4308} d_{ij} \sum_{k=0}^{S_{ij}} nutrient_{ijk} W_j}{\sum_{j=1}^{4308} \sum_{i=1}^{n} d_{ij} \sum_{k=0}^{S_{ij}} nutrient_{ijk} W_j} \times 100$$

where $i = \# \text{ food items } 1,2,\ldots,n$; $j = \text{ respondents } 1,2\ldots4308$; $k = \# \text{ servings of food items to respondent in question } 0, 1,\ldots, S_{ij}$; $S_{ij} = \# \text{ servings of the } i^{th} \text{ food by the } j^{th} \text{ respondent}; d_{ij} = 1 \text{ if the } j^{th} \text{ respondent consumed the } i^{th} \text{ food otherwise } d_{ij} = 0$; $nutrient_{ijk} = \text{ the amount of the nutrient contained in the serving } k \text{ of the food item } i \text{ to the respondent } j$; $W_j = \text{ the sample weight for the respondent } (291)$. The respondent’s weight is discussed above.

Or:

$$\frac{\text{total nutrient provided by food } i}{\text{total nutrient provided by all foods}} \times 100$$

In determination of the proportion of choline intakes below the AI, SIDE (version 1.11, Department of Statistics and Center for Agricultural and Rural Development, Iowa State University) was used to estimate usual intakes for choline using both the first and the second 24-h recall data from 10 570 respondents as described previously (292, 293). SIDE was also used to estimate the prevalence of intakes below the AI for all respondents classified according to the DRI age and sex subgroups. Survey weights were applied to this analysis using the bootstrap replication method which accounts for the unequal probability of selection as well as complex survey sampling design (287). The standard error is reported and represents the adjustment error estimate used as an estimate of the population mean.

6.4. RESULTS
Dietary Intakes and Food Sources for Folate, Vitamins B\textsubscript{6} & B\textsubscript{12} and Choline in Women of Childbearing Years

The major food sources of the one carbon nutrients folate, vitamins B\textsubscript{6} and B\textsubscript{12} and choline and the percent contribution of each food to total population intake for 4308 non-pregnant, non-lactating women between the ages of 18 to 45 y are presented in Tables 6.1 through 6.4.
Table 6.1  Top  food sources for folate intake  in Canadian women of childbearing years

<table>
<thead>
<tr>
<th>Rank</th>
<th>Food Description</th>
<th>Percent Contribution</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cereal grains and flours</td>
<td>12.32</td>
<td>12.32</td>
</tr>
<tr>
<td>2</td>
<td>pasta</td>
<td>9.20</td>
<td>21.52</td>
</tr>
<tr>
<td>3</td>
<td>rolls, bagels, pita, muffins etc</td>
<td>8.51</td>
<td>30.03</td>
</tr>
<tr>
<td>4</td>
<td>white bread</td>
<td>7.91</td>
<td>37.94</td>
</tr>
<tr>
<td>5</td>
<td>lettuce &amp; leafy dark greens</td>
<td>5.22</td>
<td>43.16</td>
</tr>
<tr>
<td>6</td>
<td>fruit juice (includes orange)</td>
<td>4.69</td>
<td>47.85</td>
</tr>
<tr>
<td>7</td>
<td>rice</td>
<td>4.27</td>
<td>52.12</td>
</tr>
<tr>
<td>8</td>
<td>fluid milk</td>
<td>3.53</td>
<td>55.65</td>
</tr>
<tr>
<td>9</td>
<td>eggs</td>
<td>2.97</td>
<td>58.62</td>
</tr>
<tr>
<td>10</td>
<td>whole wheat/grain breads</td>
<td>2.74</td>
<td>61.36</td>
</tr>
<tr>
<td>11</td>
<td>cakes, cookies, muffins (inc mix)</td>
<td>2.00</td>
<td>63.36</td>
</tr>
<tr>
<td>12</td>
<td>tea (including iced tea)</td>
<td>1.77</td>
<td>65.13</td>
</tr>
<tr>
<td>13</td>
<td>potatoes</td>
<td>1.70</td>
<td>66.83</td>
</tr>
<tr>
<td>14</td>
<td>salty snacks</td>
<td>1.60</td>
<td>68.43</td>
</tr>
<tr>
<td>15</td>
<td>other vegetables (cucumber, beets etc)</td>
<td>1.53</td>
<td>69.96</td>
</tr>
<tr>
<td>16</td>
<td>ready-to-eat breakfast cereals</td>
<td>1.35</td>
<td>71.31</td>
</tr>
<tr>
<td>17</td>
<td>whole grain/ fiber breakfast cereals</td>
<td>1.35</td>
<td>72.66</td>
</tr>
<tr>
<td>18</td>
<td>nuts and seeds</td>
<td>1.35</td>
<td>74.01</td>
</tr>
<tr>
<td>19</td>
<td>yeast</td>
<td>1.30</td>
<td>75.31</td>
</tr>
<tr>
<td>20</td>
<td>citrus fruits</td>
<td>1.28</td>
<td>76.59</td>
</tr>
<tr>
<td>21</td>
<td>tomatoes</td>
<td>1.28</td>
<td>77.87</td>
</tr>
<tr>
<td>22</td>
<td>broccoli</td>
<td>1.16</td>
<td>79.03</td>
</tr>
<tr>
<td>23</td>
<td>banana</td>
<td>1.15</td>
<td>80.18</td>
</tr>
<tr>
<td>24</td>
<td>legumes</td>
<td>1.11</td>
<td>81.29</td>
</tr>
<tr>
<td>25</td>
<td>crackers and crispbreads</td>
<td>1.05</td>
<td>82.34</td>
</tr>
<tr>
<td>26</td>
<td>beer</td>
<td>1.02</td>
<td>83.36</td>
</tr>
<tr>
<td>27</td>
<td>cheese</td>
<td>1.02</td>
<td>84.38</td>
</tr>
<tr>
<td>28</td>
<td>corn</td>
<td>0.90</td>
<td>85.28</td>
</tr>
<tr>
<td>29</td>
<td>beef</td>
<td>0.89</td>
<td>86.17</td>
</tr>
<tr>
<td>30</td>
<td>french fries, fried potatoes</td>
<td>0.82</td>
<td>86.99</td>
</tr>
<tr>
<td>31</td>
<td>soups with/without vegetables</td>
<td>0.81</td>
<td>87.80</td>
</tr>
<tr>
<td>32</td>
<td>alliums (onions, leeks, garlic)</td>
<td>0.72</td>
<td>88.52</td>
</tr>
<tr>
<td>33</td>
<td>carrots</td>
<td>0.63</td>
<td>89.15</td>
</tr>
<tr>
<td>34</td>
<td>peas and snow peas</td>
<td>0.57</td>
<td>89.72</td>
</tr>
<tr>
<td>35</td>
<td>sauces (soya, ketchup etc)</td>
<td>0.50</td>
<td>90.22</td>
</tr>
<tr>
<td>36</td>
<td>peanut &amp; other nut butters</td>
<td>0.49</td>
<td>90.71</td>
</tr>
<tr>
<td></td>
<td>Item</td>
<td>Contribution</td>
<td>Folate %</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------</td>
<td>--------------</td>
<td>----------</td>
</tr>
<tr>
<td>37</td>
<td>other fluid milk (soy, goat, etc)</td>
<td>0.48</td>
<td>91.19</td>
</tr>
<tr>
<td>38</td>
<td>yogurt</td>
<td>0.44</td>
<td>91.63</td>
</tr>
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<td>beans, fresh</td>
<td>0.43</td>
<td>92.06</td>
</tr>
<tr>
<td>40</td>
<td>other fruits (blueberry, dates, dried)</td>
<td>0.42</td>
<td>92.48</td>
</tr>
<tr>
<td>41</td>
<td>vegetable juice (includes tomato)</td>
<td>0.41</td>
<td>92.89</td>
</tr>
<tr>
<td>42</td>
<td>cabbage &amp; kale</td>
<td>0.38</td>
<td>93.27</td>
</tr>
<tr>
<td>43</td>
<td>celery</td>
<td>0.38</td>
<td>93.65</td>
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<td>44</td>
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</tr>
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<td>chicken</td>
<td>0.36</td>
<td>94.38</td>
</tr>
<tr>
<td>46</td>
<td>peppers (red &amp; green)</td>
<td>0.35</td>
<td>94.73</td>
</tr>
<tr>
<td>47</td>
<td>cauliflower</td>
<td>0.34</td>
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<td>melons</td>
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</table>

1 Contribution for folate was measured in dietary folate equivalents. Group excludes all pregnant and lactating women and includes all women aged 18-45 y. N=4308
Table 6.2  Top food sources for vitamin B₆ in Canadian women of childbearing years

<table>
<thead>
<tr>
<th>Rank</th>
<th>Food Description</th>
<th>Percent Contribution</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>chicken &amp; poultry</td>
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<td>10.88</td>
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<tr>
<td>2</td>
<td>fluid milk</td>
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<td>3</td>
<td>beef</td>
<td>6.16</td>
<td>24.22</td>
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<td>4</td>
<td>potatoes</td>
<td>5.48</td>
<td>29.70</td>
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<tr>
<td>5</td>
<td>bananas</td>
<td>5.46</td>
<td>35.16</td>
</tr>
<tr>
<td>6</td>
<td>fruit juice (includes orange)</td>
<td>3.98</td>
<td>39.14</td>
</tr>
<tr>
<td>7</td>
<td>cured meats (includes luncheon meats)</td>
<td>3.73</td>
<td>42.87</td>
</tr>
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<td>8</td>
<td>tomatoes</td>
<td>3.55</td>
<td>46.42</td>
</tr>
<tr>
<td>9</td>
<td>fried or roasted potatoes</td>
<td>3.20</td>
<td>49.62</td>
</tr>
<tr>
<td>10</td>
<td>salty snacks</td>
<td>2.34</td>
<td>54.34</td>
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<tr>
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<td>pork</td>
<td>2.28</td>
<td>56.62</td>
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<tr>
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<td>whole grain/fibre breakfast cereals</td>
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<td>58.83</td>
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<td>cheese</td>
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<td>carrots</td>
<td>1.42</td>
<td>68.93</td>
</tr>
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<td>70.27</td>
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<td>75.17</td>
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<td>24</td>
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</tr>
<tr>
<td>25</td>
<td>rolls, bagels, pita</td>
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<td>77.44</td>
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<td>26</td>
<td>lettuce &amp; leafy dark greens</td>
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<td>78.53</td>
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<td>27</td>
<td>melons</td>
<td>1.09</td>
<td>79.62</td>
</tr>
<tr>
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<td>nuts &amp; seeds</td>
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<td>81.78</td>
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<td>1.08</td>
<td>82.79</td>
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<td>83.78</td>
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<td>apples</td>
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<td>84.76</td>
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<td>33</td>
<td>peppers (red &amp; green)</td>
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<td>85.65</td>
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<tr>
<td>34</td>
<td>fruit drinks</td>
<td>0.89</td>
<td>86.43</td>
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<td>soups with/without vegetables</td>
<td>0.78</td>
<td>87.19</td>
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</tr>
<tr>
<td>38</td>
<td>peanut &amp; other nut butters</td>
<td>0.72</td>
<td>89.39</td>
</tr>
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<td></td>
<td>Description</td>
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</tr>
<tr>
<td>39</td>
<td>vegetable juice (includes tomato)</td>
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<td>yogurt</td>
<td>0.52 89.79</td>
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<tr>
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<td>other fruits (blueberries, dates, dry)</td>
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<tr>
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<td>0.38 92.14</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>corn</td>
<td>0.34 92.48</td>
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</tr>
<tr>
<td>47</td>
<td>wine</td>
<td>0.34 92.82</td>
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<td>chocolate bars</td>
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</tr>
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<td>51</td>
<td>peas &amp; snow peas</td>
<td>0.30 93.74</td>
<td></td>
</tr>
<tr>
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<td>crackers &amp; crispbreads</td>
<td>0.28 94.02</td>
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</tr>
<tr>
<td>53</td>
<td>tofu (vegetable proteins)</td>
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<tr>
<td>54</td>
<td>cabbage &amp; kale</td>
<td>0.27 94.56</td>
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<tr>
<td>55</td>
<td>mushrooms</td>
<td>0.27 94.83</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>pancakes &amp; waffles</td>
<td>0.27 95.10</td>
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</tr>
</tbody>
</table>

1 Group excludes all pregnant and lactating women and includes all women aged 18-45 y. N=4308
Table 6.3  Top food sources for vitamin B\textsubscript{12} in Canadian women of childbearing years \(^1\)

<table>
<thead>
<tr>
<th>Rank</th>
<th>Food Item</th>
<th>Percent Contribution</th>
<th>Cumulative Percent</th>
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<tbody>
<tr>
<td>1</td>
<td>fluid milk</td>
<td>24.20</td>
<td>24.20</td>
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<tr>
<td>2</td>
<td>beef</td>
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<td>41.15</td>
</tr>
<tr>
<td>3</td>
<td>cheese</td>
<td>8.86</td>
<td>50.01</td>
</tr>
<tr>
<td>4</td>
<td>cured meats (includes luncheon meats)</td>
<td>8.54</td>
<td>58.55</td>
</tr>
<tr>
<td>5</td>
<td>eggs</td>
<td>7.17</td>
<td>65.72</td>
</tr>
<tr>
<td>6</td>
<td>chicken &amp; poultry</td>
<td>6.85</td>
<td>72.57</td>
</tr>
<tr>
<td>7</td>
<td>fish</td>
<td>5.25</td>
<td>77.82</td>
</tr>
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<td>8</td>
<td>yogurt</td>
<td>3.46</td>
<td>81.28</td>
</tr>
<tr>
<td>9</td>
<td>pork</td>
<td>2.77</td>
<td>84.05</td>
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<tr>
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<td>shellfish</td>
<td>1.74</td>
<td>85.79</td>
</tr>
<tr>
<td>11</td>
<td>frozen desserts, ice cream/milk</td>
<td>1.67</td>
<td>87.46</td>
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<tr>
<td>12</td>
<td>soups with/without vegetables</td>
<td>1.43</td>
<td>88.89</td>
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<tr>
<td>13</td>
<td>meal replacements</td>
<td>0.96</td>
<td>89.85</td>
</tr>
<tr>
<td>14</td>
<td>cream</td>
<td>0.94</td>
<td>90.79</td>
</tr>
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<td>other fluid milk (soy, goat, etc)</td>
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<td>91.70</td>
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<tr>
<td>16</td>
<td>chocolate bars</td>
<td>0.84</td>
<td>92.54</td>
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<tr>
<td>17</td>
<td>instant breakfasts</td>
<td>0.68</td>
<td>93.22</td>
</tr>
<tr>
<td>18</td>
<td>beer</td>
<td>0.55</td>
<td>93.77</td>
</tr>
<tr>
<td>19</td>
<td>butter, margarine, &amp; animal fats</td>
<td>0.53</td>
<td>94.30</td>
</tr>
<tr>
<td>20</td>
<td>cakes, cookies, pies (includes mix)</td>
<td>0.44</td>
<td>94.74</td>
</tr>
<tr>
<td>21</td>
<td>cottage cheese</td>
<td>0.42</td>
<td>95.16</td>
</tr>
<tr>
<td>22</td>
<td>salad dressings</td>
<td>0.36</td>
<td>95.52</td>
</tr>
<tr>
<td>23</td>
<td>pancakes &amp; waffles</td>
<td>0.31</td>
<td>95.83</td>
</tr>
<tr>
<td>24</td>
<td>white bread</td>
<td>0.29</td>
<td>96.12</td>
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<tr>
<td>25</td>
<td>sauces (soya, ketchup, white, etc)</td>
<td>0.26</td>
<td>96.38</td>
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<td>26</td>
<td>pasta</td>
<td>0.24</td>
<td>96.62</td>
</tr>
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<td>27</td>
<td>sour cream</td>
<td>0.23</td>
<td>96.85</td>
</tr>
<tr>
<td>28</td>
<td>rolls, bagels, pita, muffins etc</td>
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<td>97.08</td>
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<tr>
<td>29</td>
<td>tofu and vegetable protein</td>
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<tr>
<td>30</td>
<td>game meat</td>
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</table>

\(^1\) Group excludes all pregnant and lactating women and includes all women aged 18-45 y. N=4308
Table 6.4  Top food sources for choline in Canadian women of childbearing years

<table>
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<tr>
<th>Rank</th>
<th>Food Description</th>
<th>Percent Contribution</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>fluid milk</td>
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<td>eggs</td>
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<td>3</td>
<td>chicken &amp; poultry</td>
<td>8.39</td>
<td>32.57</td>
</tr>
<tr>
<td>4</td>
<td>rolls, bagels, pita bread etc</td>
<td>5.91</td>
<td>38.48</td>
</tr>
<tr>
<td>5</td>
<td>cured meats (includes luncheon meat)</td>
<td>4.31</td>
<td>42.79</td>
</tr>
<tr>
<td>6</td>
<td>beer</td>
<td>4.21</td>
<td>47.00</td>
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<tr>
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<td>celery</td>
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<td>coffee</td>
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<td>54.01</td>
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<td>fish</td>
<td>2.83</td>
<td>56.84</td>
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<td>alliums (onions, leeks, garlic)</td>
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<td>tomatoes</td>
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<td>61.26</td>
</tr>
<tr>
<td>12</td>
<td>fried or roasted potatoes</td>
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<td>63.07</td>
</tr>
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<td>13</td>
<td>pasta</td>
<td>1.75</td>
<td>64.82</td>
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<td>14</td>
<td>pork, lean</td>
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<td>66.51</td>
</tr>
<tr>
<td>15</td>
<td>fruit juice (includes orange)</td>
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<td>68.18</td>
</tr>
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<td>white bread</td>
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<td>69.84</td>
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<td>potatoes</td>
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<td>71.45</td>
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<td>salty snacks</td>
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<td>73.00</td>
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<td>whole wheat breads</td>
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<td>ice cream</td>
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<td>lettuce and leafy dark greens</td>
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<td>24</td>
<td>other vegetables (cucumbers, beets etc.)</td>
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<td>81.05</td>
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<td>chocolate bar</td>
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<td>nuts &amp; seeds</td>
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<td>31</td>
<td>yogurt</td>
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<td>86.38</td>
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<td>salad dressings</td>
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<td>corn</td>
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<td>citrus fruits</td>
<td>0.64</td>
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<tr>
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<td>carrots</td>
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<td>melons</td>
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<td>91.66</td>
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<tr>
<td>peas &amp; snow peas</td>
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<td>92.05</td>
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<tr>
<td>beans, fresh</td>
<td>0.29</td>
<td>94.04</td>
<td></td>
</tr>
<tr>
<td>other fruits (blueberries, kiwi, dry)</td>
<td>0.27</td>
<td>94.31</td>
<td></td>
</tr>
<tr>
<td>cream</td>
<td>0.25</td>
<td>94.56</td>
<td></td>
</tr>
<tr>
<td>cakes, cookies, muffins (includes mixes)</td>
<td>0.25</td>
<td>94.81</td>
<td></td>
</tr>
<tr>
<td>vegetable juice (includes tomato)</td>
<td>0.23</td>
<td>95.04</td>
<td></td>
</tr>
</tbody>
</table>

1 Group excludes all pregnant and lactating women and includes all women aged 18-45 y. N=4308
Figure 6.1. Gross percentage distributions of the food groups contributing to the dietary intake of the following nutrients A) folate, B) vitamin B₆, C) vitamin B₁₂, and D) choline for Canadian women of childbearing age (18-45y). These data exclude information for all pregnant and lactating women (n=4308).
The daily mean intake (± SE) for folate for women of reproductive age was 458 ± 1.11 mg/d DFE. Table 6.1 shows the top contributors of folate in the diet, accounting for over 95% of total folate from six main food categories including: FA fortified foods; fruits, vegetables and legumes; whole grains; eggs and dairy; and, other foods. Not surprisingly, the largest contribution to total folate intake comes from FA fortified foods accounting for 45% of total folate intake (see Figure 6.1a) with cereal grains and flours and pasta being the largest contributing groups accounting for 22% of total folate intake and almost half of FA-fortified foods. Fruits, vegetables and legumes account for ~ 29% of total folate intake; combined FA-fortified foods and fruits and vegetables account approximately 74% of the total dietary folate intake in women of reproductive years.

The mean daily intake (± SE) for B₆ for women of reproductive age was 1.7 ± 0.003 mg/d. The results for the major contributors to vitamin B₆ intake are shown in Table 6.2; these top foods (fruits, vegetables and legumes; poultry and fish; eggs and dairy; red meat; whole and fortified grains; and other) account for 95% of total B₆ intake. Fruit and vegetables account for approximately 39% of total intake (see Figure 6.1b) with potatoes and bananas being the largest contributors accounting over 5% of total B₆ intake each. Poultry is the single largest contributor accounting for almost 11% of total B₆ intake. More than one-third of all food sources for B₆ are of animal origin. Interestingly, ready-to-eat fortified breakfast cereals account for less than 2% of total B₆ intake while alcohol intake from beer accounted for about 1.64%.

The daily mean intake (± SE) for B₁₂ for women of reproductive age was 3.7 ± 0.02 µg/d. The results for the major contributors to vitamin B₁₂ for women of reproductive age are presented in Table 6.3 (red meat; poultry; eggs and dairy; cured meats; fish and shellfish; and other) and account for 97% of total B₁₂ intake. Interestingly, dairy and eggs were the highest contributing food sources for B₁₂ intake at 49% (see Figure 6.1c) in women of reproductive years even though red meat is a much higher source of B₁₂ (235, 289). Fluid milk alone accounted for almost a quarter of total B₁₂ intake. Almost all of the B₁₂ food sources ~94% are from animal foods, some of the remaining 6% (e.g. grain products such as pancakes and waffles) would contain added animal foods as part of their ingredient list. Only about 2% of foods are non-animal food sources such as beer, tofu, soy milk, and fortified meal replacements.
The daily mean intake (± SE) for choline for women of reproductive age was 238 ± 0.54 mg/d. Table 6.4 presents the major contributing food sources for choline in women of reproductive age with the top foods (eggs and dairy; fruit, vegetables and legumes; meat, poultry and fish; whole and fortified grains and other) accounting for 95% of total choline intake. Dairy products and eggs are the largest contributing food sources for women of reproductive age (see Figure 6.1d), accounting for about 29% of total choline intake while fruits, vegetables and legumes contribute more than one quarter of total choline intake. Beer (found in the Other category) is also a significant source of choline (as well as containing folate and B₆) contributing more than 4% of total choline intake. Animal products account for almost half (48%) of contributing food sources for choline for women of reproductive age.

Dietary Intakes of Choline and the Prevalence of Intakes Below the Adequate Intake Level for the Canadian Population

Table 6.5 presents the mean choline intakes and the prevalence of intakes below the AI, for the Canadian population, > 1 y age, by gender and age. Children aged 1-3 y had the lowest usual mean intake (mean ± SE) at 187 ± 0.3 mg/d while males 31-50 y had the highest usual mean intake at 356 ± 3.5 mg/d. Approximately 64% of children between the ages of 1 to 3 have choline intakes below the AI (200 mg/d). As age increases for both males and females, the prevalence of intakes below the AI increases. Males 14-18 y had the second highest prevalence of intakes below 550 mg/d at 98%, while males 9-13 had a prevalence of 92%. Nearly all females older than 9 y had intakes below the AI (> 375 mg/d). Both men and women > 70 y had a prevalence rate of almost 100%.

Dietary Sources of Choline in the Canadian Population

Food sources accounting for approximately 95% of total intake for choline for the Canadian population are shown in Table 6.6 and Figure 6.2. Eggs and dairy are the largest contributors to choline intake across the population, accounting for about 42% of the estimated choline intake from various food sources. While eggs are the single largest contributor to choline intake across the population at ~ 12.5%, fluid milk (combination of all milks) is the largest contributor with 2% milk alone contributing 10.11% to total choline intake (Table 6.6).
Table 6.5  Dietary intakes of choline for Canadian population and prevalence of intakes below the AI$^1$

<table>
<thead>
<tr>
<th>Age (y) and Sex</th>
<th>N</th>
<th>Mean ± SE (mg/d)</th>
<th>AI (mg/d)</th>
<th>% below AI (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Children</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>2231</td>
<td>187 ± .33</td>
<td>200</td>
<td>64 (59, 69)</td>
</tr>
<tr>
<td>4-8</td>
<td>3346</td>
<td>214 ± .58</td>
<td>250</td>
<td>78 (72, 83)</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-13</td>
<td>2149</td>
<td>273 ± .52</td>
<td>375</td>
<td>92 (89,.91)</td>
</tr>
<tr>
<td>14-18</td>
<td>2397</td>
<td>320 ± .69</td>
<td>550</td>
<td>98 (96, 99)</td>
</tr>
<tr>
<td>19-30</td>
<td>1897</td>
<td>348 ± .85</td>
<td>550</td>
<td>95 (93, 98)</td>
</tr>
<tr>
<td>31-50</td>
<td>2747</td>
<td>356 ± 3.5</td>
<td>550</td>
<td>94 (90, 99)</td>
</tr>
<tr>
<td>51-70</td>
<td>2724</td>
<td>319 ± .68</td>
<td>550</td>
<td>96 (91, 100)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>1601</td>
<td>263 ± .5</td>
<td>550</td>
<td>99.8 (99.5, 100)</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-13</td>
<td>2043</td>
<td>225 ± .44</td>
<td>375</td>
<td>98 (95, 100)</td>
</tr>
<tr>
<td>14-18</td>
<td>2345</td>
<td>222 ± .47</td>
<td>400</td>
<td>99 (97, 100)</td>
</tr>
<tr>
<td>19-30</td>
<td>1914</td>
<td>240 ± .75</td>
<td>425</td>
<td>99 (97, 100)</td>
</tr>
<tr>
<td>31-50</td>
<td>2851</td>
<td>255 ± .56</td>
<td>425</td>
<td>98 (96, 99)</td>
</tr>
<tr>
<td>51-70</td>
<td>3406</td>
<td>241 ± .48</td>
<td>425</td>
<td>99 (98, 100)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>2768</td>
<td>212 ± .4</td>
<td>425</td>
<td>99(99, 100)</td>
</tr>
</tbody>
</table>

$^1$ Excludes all pregnant and lactating women and individuals < 1 y. N= 34 840.
<table>
<thead>
<tr>
<th>Rank</th>
<th>Food Description</th>
<th>Percent Contribution</th>
<th>Cumulative Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>fluid milk</td>
<td>23.64</td>
<td>23.64</td>
</tr>
<tr>
<td>2</td>
<td>eggs</td>
<td>12.66</td>
<td>36.30</td>
</tr>
<tr>
<td>3</td>
<td>chicken &amp; poultry</td>
<td>6.85</td>
<td>43.15</td>
</tr>
<tr>
<td>4</td>
<td>cured meats (includes luncheon meats)</td>
<td>4.48</td>
<td>47.63</td>
</tr>
<tr>
<td>5</td>
<td>alliums (onions, leeks, garlic)</td>
<td>2.36</td>
<td>49.99</td>
</tr>
<tr>
<td>6</td>
<td>coffee</td>
<td>2.23</td>
<td>52.22</td>
</tr>
<tr>
<td>7</td>
<td>beer</td>
<td>2.16</td>
<td>54.38</td>
</tr>
<tr>
<td>8</td>
<td>whole wheat breads</td>
<td>2.00</td>
<td>56.38</td>
</tr>
<tr>
<td>9</td>
<td>fruit juice (includes orange)</td>
<td>1.99</td>
<td>58.37</td>
</tr>
<tr>
<td>10</td>
<td>white bread</td>
<td>1.94</td>
<td>60.31</td>
</tr>
<tr>
<td>11</td>
<td>potatoes</td>
<td>1.93</td>
<td>62.24</td>
</tr>
<tr>
<td>12</td>
<td>rolls, bagels, pita bread, tortillas etc.</td>
<td>1.91</td>
<td>64.15</td>
</tr>
<tr>
<td>13</td>
<td>pork</td>
<td>1.84</td>
<td>65.99</td>
</tr>
<tr>
<td>14</td>
<td>tomatoes</td>
<td>1.72</td>
<td>67.71</td>
</tr>
<tr>
<td>15</td>
<td>fish &amp; shellfish</td>
<td>2.77</td>
<td>70.48</td>
</tr>
<tr>
<td>16</td>
<td>pasta</td>
<td>1.60</td>
<td>72.08</td>
</tr>
<tr>
<td>17</td>
<td>fried or roasted potatoes</td>
<td>1.57</td>
<td>73.65</td>
</tr>
<tr>
<td>18</td>
<td>frozen desserts (ice/milk)</td>
<td>1.54</td>
<td>75.19</td>
</tr>
<tr>
<td>19</td>
<td>cheese</td>
<td>1.32</td>
<td>76.51</td>
</tr>
<tr>
<td>20</td>
<td>bananas</td>
<td>1.14</td>
<td>77.65</td>
</tr>
<tr>
<td>21</td>
<td>soups with/without vegetables</td>
<td>1.06</td>
<td>78.71</td>
</tr>
<tr>
<td>22</td>
<td>other vegetables (cucumbers, beets,etc)</td>
<td>0.97</td>
<td>79.68</td>
</tr>
<tr>
<td>23</td>
<td>salty snacks</td>
<td>0.85</td>
<td>80.53</td>
</tr>
<tr>
<td>24</td>
<td>broccoli</td>
<td>0.83</td>
<td>81.36</td>
</tr>
<tr>
<td>25</td>
<td>legumes</td>
<td>0.80</td>
<td>82.16</td>
</tr>
<tr>
<td>26</td>
<td>chocolate bars</td>
<td>0.78</td>
<td>82.94</td>
</tr>
<tr>
<td>27</td>
<td>sauces (soya, ketchup etc)</td>
<td>0.77</td>
<td>83.71</td>
</tr>
<tr>
<td>28</td>
<td>corn</td>
<td>0.76</td>
<td>84.47</td>
</tr>
<tr>
<td>29</td>
<td>peanut &amp; other nut butters</td>
<td>0.72</td>
<td>85.19</td>
</tr>
<tr>
<td>30</td>
<td>nuts &amp; seeds</td>
<td>0.69</td>
<td>85.88</td>
</tr>
<tr>
<td>31</td>
<td>lettuce &amp; leafy dark greens</td>
<td>0.68</td>
<td>86.56</td>
</tr>
<tr>
<td>32</td>
<td>citrus fruits</td>
<td>0.66</td>
<td>87.22</td>
</tr>
<tr>
<td>33</td>
<td>yogurt</td>
<td>0.65</td>
<td>87.87</td>
</tr>
<tr>
<td>34</td>
<td>salad dressings</td>
<td>0.58</td>
<td>88.45</td>
</tr>
<tr>
<td>35</td>
<td>apples</td>
<td>0.56</td>
<td>89.01</td>
</tr>
<tr>
<td>36</td>
<td>carrots</td>
<td>0.55</td>
<td>89.56</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Value</td>
<td>Percentage</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
<td>-------</td>
<td>------------</td>
</tr>
<tr>
<td>37</td>
<td>celery</td>
<td>0.52</td>
<td>90.08</td>
</tr>
<tr>
<td>38</td>
<td>peas &amp; snow peas</td>
<td>0.49</td>
<td>90.57</td>
</tr>
<tr>
<td>39</td>
<td>butter, margarine &amp; animal fats</td>
<td>0.48</td>
<td>91.05</td>
</tr>
<tr>
<td>40</td>
<td>soft drinks</td>
<td>0.25</td>
<td>91.30</td>
</tr>
<tr>
<td>41</td>
<td>melons</td>
<td>0.37</td>
<td>91.67</td>
</tr>
<tr>
<td>42</td>
<td>cauliflower</td>
<td>0.37</td>
<td>92.04</td>
</tr>
<tr>
<td>43</td>
<td>cakes, cookies, muffins (includes mixes)</td>
<td>0.36</td>
<td>92.40</td>
</tr>
<tr>
<td>44</td>
<td>crackers &amp; crispbreads</td>
<td>0.35</td>
<td>92.75</td>
</tr>
<tr>
<td>45</td>
<td>rice</td>
<td>0.34</td>
<td>93.09</td>
</tr>
<tr>
<td>46</td>
<td>other fruits (blueberries, dates, dry, etc)</td>
<td>0.30</td>
<td>93.39</td>
</tr>
<tr>
<td>47</td>
<td>wine</td>
<td>0.28</td>
<td>93.67</td>
</tr>
<tr>
<td>48</td>
<td>tea</td>
<td>0.27</td>
<td>93.94</td>
</tr>
<tr>
<td>49</td>
<td>cereal grains &amp; flours</td>
<td>0.27</td>
<td>94.21</td>
</tr>
<tr>
<td>50</td>
<td>liver</td>
<td>0.24</td>
<td>94.45</td>
</tr>
<tr>
<td>51</td>
<td>beans, fresh</td>
<td>0.23</td>
<td>94.68</td>
</tr>
<tr>
<td>52</td>
<td>cream</td>
<td>0.22</td>
<td>94.90</td>
</tr>
<tr>
<td>53</td>
<td>cabbage &amp; kale</td>
<td>0.21</td>
<td>95.11</td>
</tr>
<tr>
<td>54</td>
<td>peaches &amp; nectarines</td>
<td>0.21</td>
<td>95.32</td>
</tr>
<tr>
<td>55</td>
<td>vegetable juice (includes tomato)</td>
<td>0.20</td>
<td>95.52</td>
</tr>
<tr>
<td>56</td>
<td>mushrooms</td>
<td>0.19</td>
<td>95.71</td>
</tr>
</tbody>
</table>

1 Population excludes all pregnant and lactating women and individuals < 1y.
N= 34,840
Figure 6.2. Gross percentage distributions of the food groups contributing to the dietary intake of choline to the Canadian population ≥ 1 y. This data excludes information for all pregnant and lactating women (n=34 840).
Vegetables were a significant source for choline intake with onions, leeks and garlic contributing 2.36% and coffee contributing 2.23% of intake. While meats, poultry and fish contributed approximately 18% to total choline intake, poultry was the preferred meat contributing almost 7% of total choline intake. Lean pork was the only non-cured red meat source contributing almost 2% of choline intake. An estimated 60% of the top choline food sources are derived from animal products.

6.5. DISCUSSION

Dietary contribution of total nutrients consumption is based on the nutrient content of a particular food, the portion size consumed and the frequency of consumption (291). The results of this study suggest that Canadian women of reproductive years have a widely varied intake when it comes to meeting their requirements for four critical vitamins (folate, B6, B12 and choline) in 1-C metabolism, but our results also suggest that not only do WCBY have choline intakes well below the AI but that almost all Canadians > 1 yr do not take in enough choline through dietary sources.

Changes in dietary trends and fortification legislation have changed the impact of certain foods and food groups on folate intake in North America. Prior to fortification, fruits (especially orange juice), vegetables and legumes were the primary sources of folate in the U.S. diet (88, 294) unless highly fortified breakfast cereals were consumed, in which case they were the largest contributor (200). Post fortification, breads, rolls and cereals became the most significant source of folate, with all sources of FA fortified foods contributing almost 45% of total folate in the form of FA (88). Vegetables were the next highest contributor (12.6%) with fruit, vegetables and legumes contributing about 25% of total folate intake (88). These U.S. post-fortification results are very much in line with the results of the present study which found that FA fortified foods account for up to 45% of total folate intake with fruits, vegetables and legumes contributing about 29% (Table 1). Thus, both natural and fortified folates from these two food groups account for approximately 74% of total folate intake in our study (Figure 6.1). Dietary trends can also influence what foods are consumed, especially in WCBY. In a small group of primarily university educated Canadian women, orange juice was the highest contributor to total folate intake at 11.1% while fruits, vegetables and legumes together (excluding French fries)
contributed almost 32% of total folate intake (89). Pasta was the highest fortified food product consumed at 8.8% while whole wheat, rye and other dark breads ranked higher in folate contribution (4.8%) than white bread (4.2%) suggesting emphasis on the elements of a healthy diet with higher intakes of fruits, vegetables and whole grains (89). There is evidence suggesting that a higher intake of refined grains is associated with a lower total folate intake because the quality of the diet overall is lower (181, 279).

Unlike natural folate which is found only in foods of plant origin, vitamin B₆ is found in foods of both animal and plant origin (289). In this study, fruits, vegetables and legumes are the largest contributors to total B₆ intake accounting for approximately 39% with potatoes, bananas, fruit juice and tomatoes being the top contributors (Table 6.2). The single largest contributor to B₆ intake in WCBY is poultry which accounts for almost 11% of total B₆ intake. Interestingly, there has been a shift in what meats are the largest contributors to B₆ intake. In data collected for NHANES between 1976-1980, beef steaks and roasts were the most significant source for B₆ intake at 9.1% with alcohol ranking second at 6% with potatoes also contributing 6% (62). Twenty-five years later, with the emphasis on reducing saturated fat intake, Canadian WCBY prefer chicken as their main source of B₆ with beef sources ranking 3rd contributing ~6% of total intake. A recent Australian study looked at B₆ intake in younger WCBY (mean age was ~25 y) and found that beef and veal contributed 29% of intake while Asian greens, carrots and bananas contributed 31% of intake again suggesting an increased contribution from fruits and vegetables over time (295).

Vitamin B₁₂ is more limited in the variety of sources available since it is found only in animal products with limited use as a fortificant in meal replacements and plant-based meat and dairy analogues. The results of this work suggest that almost all dietary B₁₂ for Canadian women of reproductive age is consumed in animal sources (or animal sources added to other food products such as grains) with fortified foods (meal replacement bars, soy milk, tofu) contributing approximately 2% (Table 6.3 & Figure 6.1b). Interestingly, the most commonly-consumed source of B₁₂ is fluid milk which contributes approximately 24% of total intake. Even though milk contains less B₁₂ compared to red meat, more frequent consumption would account for its ranking (235). Dairy and eggs contributes slightly more (~49%) to the total B₁₂ intake of WCBY than all animal flesh products combined (~45%) although red meat (beef and pork) are the second highest contributors overall. There are limited data looking at food sources for B₁₂.
intake but data from NHANES 2003-2006 suggests that beef, milk and cheese together contribute about ~42% of B₁₂ intake to U.S. diet with milk contributing ~17% alone (296). While the contribution of B₁₂ from fortified breakfast cereals was not included in the previous analysis, data looking at nutrient intake from foods, fortificants, and supplements suggests that fortified foods contribute 1 ± 0.03 µg/d to total B₁₂ intake thereby decreasing the prevalence of intakes below the estimated average requirement (EAR) to 3.2%, while the prevalence of inadequate intakes in Canada ranges from 5-15% depending on age and sex (59, 60). The Australian study looking at dietary intakes in young WCBY reported that caffeinated energy drinks contributed 12% of total B₁₂ intake in their sample while dairy was not a significant contributor (295). It will be interesting to discover if this recent trend is paralleled in Canadian WCBY since Health Canada does allow for the addition of B₁₂ to caffeinated energy drinks up to a daily maximum of 25 µg in the recommended consumption amount (297).

This is the first time that choline food sources and intakes have been examined across the Canadian population. Like B₆, choline is also found in food sources of both plant and animal sources as forms are both lipid (phosphatidylcholine) and water (choline) soluble (240). The relatively recent publications of the USDA database for the choline content of common foods in 2004 (updated in 2008) allowed for measuring choline intakes in various populations. We looked at food sources in WCBY specifically as well as the entire population > 1y. Canadian WBCY rely primarily on milk (~ 12%), eggs (~12%), poultry (~ 8%), rolls, bagels & pitas (~6%), cured meats (~4%) and beer (~4%) as the top contributors to their choline intake (Table 6.4). Overall, eggs and dairy (29%) are the primary contributors with fruits, vegetables and legumes (27%) closely behind in second place. Interestingly, coffee was a significant contributor at ~3% while beef in any form did not make the top foods even though 3 oz of beef steak or ground beef contain approximately 20-25% of the AI for choline for pregnant women (226). One study that looked at choline food sources in WCBY was done in New Zealand and found eggs (~13%), red meat (10%), milk (8%) and bread (5%) to be the most significant sources of total choline intake (286). The mean daily intake for choline was 316 ± 66 mg for the 125 women aged 18 to 40 y with only 16% meeting or exceeding the AI for choline (286). Our Canadian WCBY had a much lower mean (SE) daily choline intake (238 ± 0.54 mg) compared to the New Zealand women which may be related to the higher intakes of red meat and fish (4.2% vs 2.8%) in the New Zealand women. Pregnant Canadian women who ate less than 75 g of
fish a week had significantly lower choline intakes (P<0.05) and significantly lower plasma choline concentrations (P=0.023) than those who ate ≥ 150 g (298). The group looked at the choline intakes at 16 and 36 weeks gestation in 154 women in Vancouver, BC and found that mean choline intakes were much higher (383 ± 98.6 mg/d) than either our WCBY or the New Zealand women but 74% still had lower intakes than the recommended AI for women during pregnancy suggesting that efforts on informing women about the importance of choline and choline food sources during reproduction is essential (229, 298).

We also looked at choline food sources and the prevalence for intakes below the AI across the Canadian population > 1y (Table 6.5). Including young children in the overall sample for food sources did seem to influence the top ranking contributors somewhat with fluid milk contributing about 23% of total choline intake and eggs as the second higher contributor at approximately 13%. Dairy and eggs had a higher overall contribution to total choline intake of the population than it did in WCBY and the contribution from fruits, vegetables and legumes was smaller overall (Figure 6.2). Again less of an overall contribution from fruits and vegetables could be due to the inclusion of children in the sample. Interestingly, neither coffee nor beef were in the top 5 foods for choline contribution for the population, but the contribution of meat, poultry and fish was similar for the entire population as it was for the WCBY (18 and 19% respectively). While the food sources for the population as a whole are primarily animal-based sources (~60% overall) and on average these are the sources higher in choline, this does not seem to be reflected in the mean choline intakes of the population. The prevalence of intakes of choline below the AI are very high with children aged 1-3 having the lowest prevalence rate at 64%, while less than 2% of women aged 9 and older had choline intakes at or above the AI. There is little evidence looking at choline intakes at the population level as the U.S. has only been reporting population choline intakes since the 2007-2008 NHANES and prevalence of intakes below the AI have not been calculated (63). The group reported that males consumed significantly more choline than females with a mean daily intake of 396 mg compared to 260 mg for females which is not surprising as choline intake is related to caloric intake (63). Percentage contribution of food categories was also reported with eggs contributing 12%; fluid milk contributing 10%; meats, poultry and fish contributing 24%; and vegetables, potatoes and legumes contributing 7% (63). When we compare the U.S intakes to the Canadian intakes we can see a comparable intake of dairy and eggs; however, Canadian intake of fruits, vegetables
and legumes is much higher, while meat intake from all sources is lower. Eggs are an excellent source of choline and there is evidence that plasma choline concentrations can be predicted by egg consumption (skim milk, coffee and beer also had a small but significant positive effect, P=0.03) with increases of 0.16 µmol/L for every quartile of food intake (299). Evidence from a number of studies looking at primarily middle-aged adults reported choline intakes for males and females with intakes for males ranging from 302-372 mg/d and female intakes ranging from 271-314 mg/d with the majority of subjects in all studies being below the AI for choline (300-302). The dietary intake evidence does suggest that dietary intake of choline is declining as people avoid foods high in cholesterol (such as eggs) or saturated fat (such as red meat) both of which are excellent sources of choline (303).

By definition, the AI is inherently limited when applied to assess nutritional adequacy; it cannot be used to determine adequacy of intake if the group mean intake is below the AI (304). As per the Institute of Medicine, AIs are established when there is insufficient scientific evidence regarding nutrient intake to determine an Estimated Average Requirement (EAR) but they are thought to meet or exceed the amount required for adequacy in a health population (304). With regards to choline, the AI is based on a single experiment that was conducted on 16 healthy males approximately 29 years of age who were fed a control or deficient diet of choline for 3 weeks with the prevention of alanine aminotransferase abnormalities (a marker of liver dysfunction) as the outcome (42). AI values for remaining population groups (with the exception of 0-6 mths) were extrapolated from this evidence. Factors such as genetics, utilization, bioavailability, and the estrogen sensitive de novo synthesis of choline from the phosphytidylethnalamine N-methyltransferase pathway were not considered in developing the AIs, but can affect individual dietary choline requirements thereby limiting the applicability of the AI even further (303). It is to be hoped that more recent research looking at these genetics, sex differences and so forth, will be utilized in order to determine a more accurate reflection of choline requirements across the population.

There is little doubt that the FA fortification policies in North America and the recommendations for FA supplement use have had a positive effect on reducing NTD (6, 105). However, there is a growing body of evidence suggesting other methyl nutrients (B₆, B₁₂ and choline) may also be related to NTD and other defects (245, 249, 256). Further, that any dysfunction in the interactions between the various methyl vitamins and amino acids can also
lead to cognitive impairment; affect lipid transit and storage; and affect methylation metabolism impacting on post-translational modification of proteins, DNA methylation, and the synthesis of such compounds as hormones, carnitine, creatine and phosphatidylcholine (20, 57, 283). Choline has importance beyond its role as a methyl nutrient as it is required for neurotransmitter synthesis, cell-membrane signalling and is critical for normal development in the fetal brain during gestation (2, 217). As our understanding of the roles of the methyl nutrients have in maintaining 1-C metabolism increases, it becomes clear that optimal intakes of these crucial nutrients is necessary not only during the reproductive years for women but throughout the lifecycle. Evidence does suggest that dietary intakes of methyl vitamins is adequate in the majority of WCBY and the population in general with inadequate dietary intakes between 3-25% for B₆, B₁₂ and folate (59, 60). The evidence suggests otherwise for choline with the majority of the populations across a number of different studies having intakes well below the AI reinforcing the results of our study. There has been so much evidence and education about the importance of folate that we have lost sight that all the methyl nutrients are important; indeed, evidence is suggesting that it is the interrelationship between these nutrients on methylation patterns that has more impact than the overage or deficiency of one nutrient within the cycle (57). Since choline is not typically included in multivitamin formulations (226) it behooves researchers and clinicians to emphasize the importance of choline and its role in both reproductive health and overall general health. Further, changes in dietary trends (e.g., reducing saturated fat, vegetarianism, and processed foods) can also put women further at risk of inadequate intakes especially with B₁₂ and choline which are concentrated in animal food sources, unless care is taken to educate about appropriate food sources and quantities are consumed.

This study is limited by the current restrictions of the choline information contained in the 2010 version of the CNF. This is based on the USDA Database for the Choline Content of Common Foods and contains information on only 634 foods while the CNF contains information on 5708 foods. This disparity limits our ability to capture the complete information on choline dietary intake. Evidence from other studies does suggest that underreporting choline intake from lack of information on food content is a consistent problem (300-302). Further, since many foods have been added to the CNF since the CCHS 2.2 survey was done, the CNF may contain nutrient information for foods that were not consumed during the time period of the survey further
reducing the ability to capture choline intake. As the choline database information improves, our ability to capture dietary choline intake will also improve.

In conclusion, a wide variety of food sources are consumed to provide intake of crucial methyl nutrients; however, the heavy dependence on folic acid fortified foods or animal-sourced foods, especially for choline, B₁₂ and B₆, may increase the likelihood of not meeting requirements when WCBY avoid certain foods or food groups to reduce intake of cholesterol and saturated fats, or processed and refined foods. The prevalence of intakes below the AI for the Canadian population and specifically for WBCY is extremely high but we are limited in our ability to infer whether this is a cause for concern due to the inherent limitations of the AI. However, given the importance of choline and the other methyl nutrients to 1-C metabolism it is important to ensure adequate intake of these nutrients especially during the reproductive years.
One carbon (1-C) metabolism supplies the carbon units for DNA/RNA synthesis and produces S-adenosylmethionine (SAM) the primary methylation molecule in over 40 different reactions in the body (92). The increased biosynthesis of DNA/RNA and the metabolism of key amino acids to support the growth and development of the uteroplacental organs and fetus is the primary reason for the increase in methyl vitamin requirements, including folate, vitamins B₆ and B₁₂, and choline, during pregnancy (3). As the primary donor of carbon units, folate has been studied the most extensively. The focus for folate during reproduction has shifted somewhat to focus on the role of folate in reducing neural tube defects (NTD) and other birth defects (3). Evidence has shown that adequate folate status, and to a lesser extent the other methyl vitamins, during reproduction, not only prevents against pregnancy-induced methyl vitamin deficiencies but promotes normal metabolic function of 1-C metabolism (104). While NTD are multifactorial and the mechanism for folate-related NTD remains unclear, it is generally acknowledged that any dysregulation that affects one carbon (1-C) metabolism has the potential to increase the risk for NTD and other birth defects (104). Thus is it crucial to understand what factors can influence folate status or the function of 1-C metabolism not only during pregnancy but during the reproductive years of women given that up to 50% of pregnancies are unplanned (11). Evidence suggests that between 12-25% of Canadian women of childbearing years (WCBY) have inadequate intakes of vitamins B₆, B₁₂, and folate from dietary sources (59). While there are currently no data examining choline intakes in Canadian WCBY (prior to this thesis), data from the U.S. indicates choline intakes have been declining in recent years (303). The overarching goal of this thesis was to examine factors related to folate status in women during their childbearing years and to understand which groups of food in the Canadian food supply contribute to dietary folate and the other 1-C vitamins B₆, B₁₂ and choline.

In the first study, entitled “Neither Folic Acid Supplementation nor Pregnancy Affects the Distribution of Folate Forms in the Red Blood Cells of Women” we compared the distribution of folate forms in RBCs of pregnant women and non-pregnant women and among non-pregnant women consuming 0, 1 and 5 mg FA in supplemental form to test if there is a physiological shift in 1-C metabolism to accommodate the rapid rate of anabolic activity during pregnancy. Further we were interested to see if varying doses of FA in supplemental form
influenced the intracellular distribution of folate in 1-C metabolism in WCBY. We found there was no difference in the distribution of folate forms in RBCs in the pregnant women taking 1 mg FA and the non-pregnant women taking 1 mg FA suggesting that there is little evidence of a physiological shift early in pregnancy to accommodate increased DNA/RNA synthesis. Further, while there was a significant difference in total RBC folate concentrations in non-pregnant women taking 1 mg or more of FA compared to those not taking any supplements there is little evidence that FA in high concentrations alters the distributions of the folate forms in RBCs, suggesting no metabolic adaptation to pharmacologic doses of FA.

The results of our first study showing no evidence of preferment for folate towards purine and pyrimidine synthesis for erythropoiesis during pregnancy; contrary to our original hypothesis of anticipating a shift in folate metabolism from re-methylation of homocysteine to purine and pyrimidine biosynthesis demonstrated by an increase in non-methyl folate forms. Evidence of physiological shifts in 1-C metabolism including in RBCs (as reflected by alterations in folate form distributions) has been described in the literature in association with dietary deficiencies, environmental exposures and abnormal anabolic conditions such as cancer (45-49). We did not find evidence of a physiological shift toward biosynthesis in RBC folate, which is reflective of metabolism at the time of RBC formation; however, it is possible that the shift occurs in other tissues which we did not examine. Further, the sample size in this study was small and the small differences noted in this study were not significant in part due to this limitation.

In the second study entitled “An Enhanced List of Socio-demographic, Dietary and Lifestyle Factors Does not Identify Women with RBC Folate Concentrations Associated with Protection against Neural Tube Defects” we tested a comprehensive list of socio-demographic and lifestyle factors (age, education, income, ethnicity, country of birth, smoking, alcohol use), genetic polymorphisms of the C677T MTHFR gene, specific information about FA and B12 supplement use, (including information about dose and frequency of use) as well as dietary intakes (both total folate intake and dietary sources of folate) all associated with NTD risk or folate status associated with NTD protection among WCBY. The list of variables was derived from epidemiological evidence, both pre- and post-FA fortification studies, of factors related to NTD risk but the more recent post-fortification study did not include all previously associated variables and lacked information on genetic polymorphisms and details related to FA supplement use. Our goal was to develop a clinical tool for use by clinicians to identify women who are
likely to have NTD-protective folate status (≥ 906 nmol/L) as direct measurement of RBC folate concentrations is not encouraged. We found that women using FA supplements at least twice a week were 12.6 more likely to have RBC folate concentrations associated with NTD protection while an alcohol intake < 1 drink/wk increased the risk of not reaching an NTD protective RBC folate concentration by 64%. The final predictive models, from the ROC analysis, suggest that a FA supplement dose of 200 µg/d (or a multivitamin supplement taken every other day), and taking into consideration alcohol intake and ethnicity is predictive of a RBC folate concentration associated with NTD protection 72-73% of the time. The results of this study suggest that while FA containing supplements are the best method to ensure that WCBY have a folate status associated with NTD prevention, they do not necessarily need to take FA daily to achieve this goal, but rather a multivitamin taken every other day (or an equivalent 200 µg dose taken daily) is sufficient for healthy women to achieve an NTD-protective folate status. Factors such as alcohol use and ethnicity should also be questioned by clinicians, as they may negatively impact the ability to achieve an NTD protective folate status since alcohol has been shown to have complex relationship to folate status and a number of ethnicities are associated with an increased risk of NTD and a lower folate status (187, 190, 278).

The results of our second study demonstrated, contrary to our hypothesis, that a comprehensive list of variables does not improve our ability to predict if a woman is more likely to have RBC folate concentrations associated with NTD protection. Instead, our results indicate that the strongest predictive model includes FA supplements, and takes into consideration alcohol use and ethnicity. While the results of study 1 indicate that varying levels of FA supplementation have not been shown to alter the distribution of folate forms in 1-C metabolism suggesting that they do not have a significant metabolic effect, the results of both study 1 and 2 do show that FA supplement intake can significantly increase RBC folate concentrations. While FA fortification and supplementation are both beneficial in WCBY in reducing NTD (6, 14, 92) there has also been a corresponding increase in RBC folate concentrations. On average only 22-25% of WCBY have concentrations below the range associated with NTD protection, but a high proportion of pregnant women have concentrations above the cut-off associated equivalent to the 97% percentile of the U.S. population prior to fortification (16, 17, 40). With the conflicting evidence suggesting that high intakes of FA may exacerbate the effects of low B\textsubscript{12} status (20, 21) and may alter DNA methylation patterns or have adverse fetal outcomes (23, 29-31, 34) then the
results of the second study suggesting that a multivitamin taken every other day is sufficient to achieve an NTD protective folate status is helpful to reduce the number of women with very high RBC folate concentrations.

In the final study entitled “Sources of folate, vitamins B\textsubscript{6} and B\textsubscript{12}, and choline among Canadian women of childbearing age: Do women of childbearing age approach adequate levels for choline” we examined the predominant dietary sources of folate, vitamins B\textsubscript{6} and B\textsubscript{12} and choline consumed by Canadian WCBY. As a secondary objective, we calculated the choline intake in Canadian WCBY and determined the prevalence of intakes below the AI. Along with folate, vitamins B\textsubscript{6} and B\textsubscript{12}, and choline are the other main methyl vitamins involved in 1-C metabolism and evidence suggests that not only are these vitamins independently associated with NTD risk (247, 249, 256) but that dietary intake of these nutrients may not be sufficient for some WCBY and issues of dietary quality and intake would increase the number of WCBY with insufficient intakes (59, 60). We found that Canadian WCBY have a widely varied intake of food sources to meet their dietary intakes with approximately 45\% of dietary folate coming from FA fortified food sources; 37\% of B\textsubscript{6}, 94\% of B\textsubscript{12} and 40\% of choline coming from animal-based food sources; while 29\% of folate, 39\% of B\textsubscript{6} and 27\% of choline come from fruits, vegetables and legumes. Further, we found that nearly all Canadian WCBY have choline intakes below the AI. This is the first time that both choline intake and choline food sources have been examined in Canadian WCBY. These results suggest that variety of diet is key to achieving adequate intake of the other 1-C vitamins. It also appears that choline intake is problematic in Canadian WCBY. This may be an area of concern for health care providers given how crucial choline is for normal fetal neurological development (226).

While the results of the second study did not show that dietary intake of folate had any real impact on folate status, in the absence of FA containing supplements then dietary folate intake becomes the primary source of folate. Given that less than one-third of WCBY use multivitamins or FA containing supplements at any given time (56, 60), the majority of women of childbearing age are relying on dietary intake, either from natural or fortified sources to meet their nutrient requirements for folate and the other 1-C nutrients. Study 3 is the first to look at food sources for folate, vitamins B\textsubscript{6} and B\textsubscript{12} and choline for Canadian WCBY. The evidence suggests that variety is key in ensuring an adequate intake of these vitamins. More than 50\% of folate intake comes from FA fortified foods and about 20\% from fruits, vegetables and legumes.
while prior to fortification, fruits, vegetables and legumes contributed approximately 38% of total folate intake in the U.S population (294). However, even with 45% of total folate intake coming from FA fortified foods approximately 25% of Canadian WCBY still have folate intakes below recommendations and between 12-13% for B₆ and B₁₂ (59). Diet quality and variety do seem to influence whether or not dietary recommendations are met. A more ‘western’ style diet high in refined grains, starchy vegetables, meat and fat has significantly lower intakes (p< 0.05) of folate, B₆ and B₁₂ compared to a ‘more healthful diet’ defined as high intakes of fruit, vegetables, whole grains, eggs, nuts, legumes and dairy (181). The advent of convenience foods, dietary and social trends (e.g. low-carb, vegetarian/vegan, reduce red meat intake), food availability and access to food could all potentially impact diet quality and micronutrient intake in WCBY (305). This is especially crucial for B₆, choline and B₁₂ given that our results suggest that animal-based products contribute between 37-94% of total nutrient intake depending on the nutrient in question. Choline seems especially vulnerable as our results suggest that almost all WCBY do not meet the AI for choline intake. This is reinforced by other studies which found that on average, WCBY have choline intakes below the AI (229, 286) and these data are repeated when looking at the U.S. population as a whole (63). However, given the limitations of the AI we cannot determine if this is cause for concern as it cannot be used to determine adequacy of intake.

While fortification and supplementation strategies do play a role in improving nutrient intake, certain nutrients, such as choline may not be viable for widespread distribution through the food supply due to adverse effects such as the formation of tremethylamine which imparts a fishy body odor as well as symptoms of vomiting, sweating, gastrointestinal side effects and hypotension from higher intakes (306). It is suggested that education on food-based approaches is the best approach to improve overall diet quality and nutrient intake with judicious use of fortified foods and supplements when available food sources do not meet requirements (64, 305, 307). A food-based approach has the added benefit of limiting supraphysiologic intakes of nutrients such as folate which may cause perturbations in 1-C metabolism and exacerbate signs of B₁₂ deficiency or sub-optimal status, altering DNA methylation which could impair hepatic lipid metabolism, influence cancer development as well as epigenetic effects (20, 57, 106, 126, 283, 284).
7.1. LIMITATIONS

Both Study 1 and 2 are limited by the sample size used in each study. In Study 1 this may have prevented us from seeing significance in the small differences noted between groups for some of the folate forms, but whether these small differences are clinically relevant and warrants further study is unknown. In our second study, the reduced power in the binomial regressions suggests that the sample size may have been too small to adequately explore the risks associated with higher intakes of alcohol (187, 193), the influences of diet, especially the relationship with fruit and vegetable intake (54) and ethnicity (187) all of which have been seen in the literature. This study may also have been limited by the high proportion of students in our sample. Study 3 is limited by the incompleteness of the choline information contained in the 2010 Canadian Nutrient File which was used to derive the choline values in the foods listed in the Canadian Community Health Survey (CCHS) 2.2 which limits our ability to capture all the information on choline dietary intake. This underreporting of choline intake due to the limited information available is a consistent problem in the literature at this time (300-302). Further, the limitations in the information used to determine the AI for choline suggests that more research is needed to better capture requirements for women of childbearing years.

7.2. OVERALL CONCLUSIONS

Each of the three thesis studies examined various factors that can influence 1-C metabolism in WCBY. The first study looks at the question of whether or not the anabolic state of pregnancy or regular use of FA containing supplements can in any way alter folate forms distributions in the RBCs of women. We found that neither pregnancy nor FA supplementation influenced the folate form distributions in RBC of women indicating that there was no metabolic adaptation from either factor. The second study examined the impact of various socio-demographic, lifestyle, genetic and dietary factors on folate status and more specifically if they enabled women to achieve a folate status associated with NTD protection. We found that taking a FA-containing supplement at least twice a week and considering alcohol intake and ethnicity provides the best model for predicting if WCBY achieve a folate status associated with NTD protection. The last study examined the predominate dietary sources of folate, vitamins B₆ and B₁₂, and choline in Canadian WCBY and calculated the total choline intake in WCBY as well as
determining the prevalence of intake below the AI. We found that WCBY rely on a widely varied diet for their intake of these methyl vitamins but that choline intake may be problematic as almost all WCBY had intakes below the AI. By definition the AI is expected to meet or exceed the true requirements for all members of a healthy population therefore the results of this study suggest that choline intake for WCBY is an issue (304).

The evidence from this thesis suggests that FA supplementation is the strongest factor influencing folate status and does not significantly influence the distribution of folate forms in 1-C metabolism, but that daily consumption is not necessary to improve folate status. Further, in the absence of supplement use, variety in dietary intake is key to ensuring adequate intake not only of folate but all 1-C vitamins. Choline intake may be an issue in Canadian WCBY and may require food-based strategies to improve intake. Therefore the focus for prevention needs to expand to monitor intake of all 1-C vitamins, not just folate, to ensure optimal pregnancy outcomes and long-term health.

7.3. FUTURE DIRECTIONS

The body of this work has answered some questions but left many more to be answered. In study 1, while there were no significant differences in the distribution of folate forms between groups there were some small differences of note. The number of women with measurable concentrations of 5,10-methenyltetrahydrofolate was higher those who were taking either 1 or 5 mg FA compared to those not taking any supplements and, second, there were more pregnant women with measurable concentrations of 5-formyltetrahydrofolate than any of the non-pregnant women taking 0, 1 or 5 mg FA. It remains to be determined if these small differences are clinically relevant and worth future exploration.

It is the work in the last study which provides the most scope for future work. The analysis of food sources in WCBY provides a significant starting point to examine the impact of dietary manipulation or evolution of eating practices (e.g. vegetarianism) or food insecurity on food source choices, especially considering the impact this could have on B_{12} and choline status. These nutrients are critical to cognitive function both in adults and, more critically, in fetal development. Currently, there is little information about choline intakes in the Canadian population nor is there much information about the effect of dietary restrictions, either voluntary...
or involuntary, on B\textsubscript{12} or choline status in WCBY. The use of the dietary intake data in the CCHS 2.2 and the upcoming CCHS scheduled to be conducted in 2015, which will again include dietary intake data, will provide population-based data to explore these areas. Smaller community-based or clinical studies can further refine the information gathered from the population evidence. The increasing availability of convenience foods, growing dietary and social trends (e.g. low-carb, vegetarian/vegan, reduce red meat intake) especially with women of reproductive age being the largest fraction with these food practices, food availability and access to food could all potentially impact diet quality and micronutrient intake. Understanding what food choices women are making enables public health and community researchers to tailor the information and guidelines to optimize nutrient status. This is especially important when considering some of the recent evidence linking low B\textsubscript{12} intake with increased cognitive dysfunction and low choline intake with heart disease.

There are still gaps in our body of knowledge in women’s health both in terms of promoting optimal nutrient status during the reproductive years and beyond. Collecting evidence both from the population data as well as smaller, more focused epidemiological- and community-based studies to examine what women of childbearing years understand in regards nutrition, supplement use, and what food and supplement practices they use would enhance our knowledge and highlight the strengths of the current nutrition policy recommendations as well the limitations.
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Research Consent for Genetic Testing

**Title of Research Project:** Does pregnancy or high folic acid supplementation or both alter erythrocyte folate forms in women?

**Investigators:**

- **Principal Investigator:** Deborah L. O'Connor PhD, RD  
  (416) 813-7844
- **Co-investigators:**  
  Dr Tsunenobu Tamura  
  (205) 934-7478  
  Dr Robert Pawlosky  
  (301) 443-9029  
  Brenda Hartman-Craven RD, PhD (cand)  
  (416) 813-7654 ex 6175

**PhD supervisor:** Dr O’Connor

**General Section**

We will also use your blood sample to determine whether you have a genetic mutation in an enzyme called methylenetetrahydrofolate reductase (short form MTHFR). The mutation is called MTHFR C677T. The MTHFR enzyme is required for your body to most efficiently process the folate in your food, but even if this enzyme is mutated you will still be able to process folate to some degree. This mutation is common and has been shown to be present in about 10-15% of those who are of Caucasian and Asian decent, approximately 20% of those of Italian heritage, and between 2-3 percent of those of African decent. About 50% of people have a milder form of this mutation.

If you have the full mutation, you will have a lower activity of the MTHFR enzyme in your body. This can affect the way folate is used and can be of concern when you have a low amount of folate in your blood. This can further increase your risk for neural tube defects, spina bifida, cleft palate or heart defects in a baby during pregnancy and may also increase your risk for certain cancers and heart disease if your folate intake is low.

By performing this test, we will know if you have the full or milder form of the mutation. We will then use this information to help us understand your blood folate levels.

I confirm that the __Brenda Hartman-Craven__ has explained the genetic tests that I am about to have done with respect to _C677T MTHFR__, and that any questions that I have asked have been [professional's name] [name of genetic condition]
answered to my satisfaction. The discomforts, consequences and possible risks associated with these tests have been explained to me. I understand that it is my choice whether or not to have this testing. Results of this test will be explained to me and I understand that this information may be shared, if necessary, with professionals involved in my medical care, including our family physician. I have been assured that records relating to me or my child and the care that we received will be kept confidential, and that no information will be released or printed that will reveal my without my permission or unless required by law.

I understand that although genetic testing is usually accurate, as with all testing some inaccuracies may occur. Also genetic testing is ongoing and new research may mean that the interpretation of the test results may change over time. On occasion, in the process of testing for one genetic condition, another genetic alteration may be identified. Such findings would be reported to your health care provider to discuss with you.

I understand that it is my responsibility to notify the ___Clinical Dietetics___[department name] department of any change of address, and to check with the department for updated genetics information and counseling that I feel I may need, for example in making decisions about a pregnancy.

**Closed Consent:**

Closed consent means that any tissue or DNA obtained from me will be analyzed and then destroyed. Specifically, I give my consent for a blood/tissue sample to be taken for testing related only to the verification of the C677T MTHFR genotype; this testing will be undertaken in a accredited clinical service laboratory and/or a research laboratory and that the sample and any DNA extracted from it will be destroyed once the results of the testing are available. I also understand that if I want any further genetic testing to be done in the future, I will need to have another sample taken from me.

**Signature:** ______________________  
**Date:** ______________________

**Witness:** ______________________  
**Date:** ______________________
Appendix B

Research Consent Form

Title of Research Project:
Does pregnancy or high folic acid supplementation or both alter erythrocyte folate forms in women?

Investigator(s):
Principal Investigator: Deborah L. O’Connor, PhD, RD (416) 813-7844
Co-Investigators:
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Dr Robert Pawlosky (301) 443-9029
Brenda Hartman-Craven, RD PhD (cand) (416) 813-7654 ex 6175

You are being invited to participate in this research study. The researcher will explain the purpose of the study. He or she will explain how the study will be carried out and what you will be asked to do. The researcher will also explain the possible risks and possible benefits of being in the study. You should ask the researcher any questions you have about any of these things before you decide whether you wish to take part in the study. This process is called informed consent.

This form also explains the research study. Please read the form and talk to the researcher about any questions you may have. Then, if you decide to be in the study, please sign and date this form in front of the person who explained the study to you. You will be given a copy of this form to keep.

Purpose of the Research
Folate, also called folic acid, is a B vitamin. Folate is an important vitamin found in many foods, especially green leafy vegetables, orange juice, broccoli and wheat germ. Our bodies use this vitamin to make sure our cells grow properly and produce new cells. Low amounts of folate in the diet have been connected to birth defects in the baby such as neural tube defects. Health Canada recommends that all women of childbearing years ensure that they get enough folate from food and supplements (if needed) around the period of conception and for the first trimester. Therefore, women need folate before and during pregnancy to make sure that the baby will grow and develop properly.
Folate will change form slightly in the body depending on what it is being used for. In this study we are interested in examining if the most frequent form of folate in the blood of pregnant women is different from the most frequent folate forms in non-pregnant women who are either taking or not taking supplements.

We are also interested in examining whether or not how the blood samples are prepared will influence the concentrations of the different forms of folate. So we want to compare two common methods used by scientists and see if there is a difference.

**Description of the Research:**

We will study 24 women (6 will be approximately 30-36 weeks pregnant and taking 1 mg of folic acid, 12 non-pregnant and taking folic acid supplements [either 1 mg or 5 mg of folic acid] and enrolled from an ongoing study, and 6 will be non-pregnant and taking no folic acid supplements).

If you are eligible to participate in the study you will asked to come to the Clinical Investigation Unit (5D12 Atrium) at the Hospital for Sick Children in the morning. We would ask you that not eat or drink anything (except for water and folate-devoid beverages such as ginger, lemon balm and rose hip herbal teas which we will provide if desired) from about 10 pm the night before. We would also ask that you refrain from taking any of your usual supplements either the night before or the morning of your visit. We will then take about 10 millilitres of blood from a vein in your arm. This blood sample will be used to measure the total amount and form of folate in your blood. This blood sample will also be used for DNA testing to determine whether you have a common genetic mutation (see Research Consent for Genetic Testing form which will also require your signature) which can alter the amounts of the forms of folate in your blood. If you are found to have this mutation we will contact you and inform you of the results.

We will also ask you some personal information questions. We will ask you about:

- How old you are
- How healthy you are in general
- how many times you have been pregnant
- if currently pregnant, your due date and how far along you are
- how often you smoke or drink alcohol
- if you are using oral contraceptives and what level of dosage is the prescription
- if you use supplements, what kind and how often you take them
- what influences your ability/desire to use supplements
• why you may use supplements

**Potential Harms:**
There may be a small amount of bleeding when blood is taken from a vein and there may be slight discomfort and bruising or redness that will usually disappear in a few days.

**Potential Benefits:**
There is no direct health benefit to you for participating in this study. However the information we learn from this study will help us better understand how folate is used during pregnancy. The information collected will help us better understand how women use vitamin and mineral supplements especially during pregnancy.

**Confidentiality:**
Confidentiality will be respected and no information that discloses the identity of the subject will be released or published without consent unless required by law. This legal obligation includes a number of circumstances, such as suspected child abuse and infectious disease, expression of suicidal ideas where research documents are ordered to be produced by a court of law and where researchers are obliged to report to the appropriate authorities. For your information, the research consent form will be inserted in the patient health record. Health records identifying the patient may be given to and inspected by the Sickkids Clinical Research Monitors. By signing this consent form, you agree to let these people look at your study records if necessary. We will put a copy of this research consent form in your study records. We will give you a copy for your files. The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members.

Following completion of the research study, the data will be kept as long as required and then destroyed as required by Sick Kids policy. Published study results will not reveal your identity.

**Reimbursement:**
We will pay for all your expenses for taking part in the study. For example, we will pay you $25.00 dollars for your meals, babysitters, parking, and getting to and from Sick Kids. If you stop taking part in our study, we will pay your for expenses for taking part of the study so far.

**Participation:**
It is your choice to take part in this study. You can stop at any time. The care you get at Sick Kids will not be affected in any way by whether you take part in this study. You will be given access to the results of the study once they become available. New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in the study.

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study. If you become ill or are harmed because of study participation, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The study staff, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

**Sponsorship:**
The sponsor and/or funder of this research is Mead Johnson/Heinz/Weston Endowment for Nutrition/Metabolism Research.

**Conflict of Interest:**
I, and the other research team members, have no conflict of interest to declare
**Consent:**

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my health care at Sick Kids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.
7) I have read and understood pages 1 to _____of this consent form. I agree, or consent, to take part in this study.

_________________________________
Printed Name of Subject & Age

______________________________
Subject’s signature & date

_________________________________
Printed Name of person who explained consent

______________________________
Signature & date

_________________________________
Printed Witness’ name (if the subject/legal guardian does not read English)

______________________________
Witness’ signature & date

If you have any questions about this study, please call Brenda Hartman-Craven at 416-813-7654 ex 6175.

If you have questions about your rights as a subject in a study or for information on whom to contact in the event of injuries during a study, please call the Research Ethics Manager at 416-813-5718.
Appendix D

Research Consent Form

Title of Research Project:

Examining the folate status of Canadians

Investigator(s):

Principal Investigator
Deborah L. O’Connor PhD, RD
(416) 813-7844

Co-Investigators:
Mark Tremblay, PhD
(613) 737-7600 ext. 4114

Lise Dubois, PhD, RD
(613) 562-5398

Cynthia Colapinto, MSc, RD, PhD (c)
(613) 737-7600 ext.4191

You are being invited to participate in this research study.

The researcher will explain the purpose of the study. He or she will explain how the study will be carried out and what you will be asked to do. The researcher will also explain the possible risks and benefits of being in the study. You should ask the researcher any questions you have about any of these things before you decide whether you want to take part in the study. This process is called informed consent.

This form also explains the research study. Please read the form and talk to the researcher about any questions you may have. Then, if you decide to be in the study, please sign and date this form in front of the researcher. You will be given a copy of this form to keep.

Purpose of the Research

Folate, also called folic acid, is a B vitamin. Folate is an important vitamin found in many foods, especially green leafy vegetables, orange juice, broccoli and wheat germ. Our bodies use this vitamin to make sure our cells grow properly and make new cells. Low amounts of folate in the diet have been linked to birth defects in the baby. In this study, we are also interested in looking at whether Canadians and Americans have similar red blood cell folate using data from two sources the Canadian Health Measures Survey (CHMS) and the National Health and Nutrition Examination Survey (NHANES). These surveys measure folate differently. In order to compare the folate status of the Canadian population to the American population, we need to find out more about this difference. In this study, we will use the blood collected to measure red blood cell folate. Two ways to measure red blood cell folate will be used. We will then create an equation that will help explain the difference in results between...
the two ways of measuring red blood cell folate.

**Description of the Research:**

We will study 200 people. If you are eligible to participate in the study you will asked to come to the Clinical Investigation Unit (5D12 Atrium) at the Hospital for Sick Children in the morning. Participating will take around 30 minutes. Please do not eat or drink anything (except for water and beverages that do not contain folate, such as ginger, lemon balm and rose hip herbal teas) from about 10 pm the night before. Also, please do not take any of your usual supplements either the night before or the morning of your visit.

We will then take about 10 millilitres or 2 teaspoons of blood from a vein in your arm. This blood sample will be used to measure the total amount and form of folate in your blood. This blood sample will also be used for DNA testing to determine whether you have a common genetic variant which can change the amounts or forms of folate in your blood. The genetic testing is explained further in the attached Genetic Testing consent form. If you are found to have two copies of this variant (homozygous), or are found to be folate deficient, we will contact you and inform you of the results. Repeat testing in a clinical laboratory will also be recommended to confirm these results.

We will also ask you the following personal questions:

- How old you are
- Place of birth and cultural/racial background
- Your income and education level
- Whether you smoke
- How often you drink alcohol
- If you are using oral contraceptives and what level of dosage is the prescription
- Have you taken antibiotics in the last two weeks
- How healthy you are in general
- Pregnancy status
- What dietary sources of folate you eat and how often
- If you use supplements, what kind and how often you take them and why

**Potential Harms:**

There may be a small amount of bleeding when blood is taken from a vein and there may be slight discomfort and bruising or redness that will usually disappear in a few days. There is also a small risk for infection, but efforts will be made to reduce this risk. The nurse will try to take blood a maximum of three times, but if are upset by this at any time you can stop and leave without any penalty. You can also ask for a numbing cream to decrease any pain.

**Potential Benefits:**
There is no direct health benefit to you for participating in this study. However what we learn from this study will help us understand how the folate status of Canadians compares to that of Americans. This will help us inform important policies, including folate supplementation recommendations for women who may become pregnant. Also, the creation of an equation for comparing the results of folate measured in two different ways will allow other researchers to compare folate results.

Confidentiality:

No information that discloses your identity will be released or published without consent unless required by law. This legal requirement includes a number of situations, such as suspected child abuse, infectious disease or expression of suicidal ideas where research documents are ordered to be provided by a court of law and where researchers must report to the appropriate authorities. By signing this consent form, you agree to let these people look at your study records if necessary. We will put a copy of this research consent form in your study records. We will give you a copy for your files. Health records identifying the patient may be given to and inspected by the SickKids Clinical Research Monitors. The SickKids and Children’s Hospital of Eastern Ontario Research Ethics Boards may have access to your study records for auditing purposes. The data from this study will be stored in a secure, locked location. Only members of the research team (and maybe those people described above) will have access to the data. This could include external research team members. At the end of the research study, the data will be kept for 7 years after publication and then destroyed as required by Sick Kids policy. Published study results will never reveal your identity.

Reimbursement:

We will give you $25 for your expenses for taking part in the study. For example, your meals, babysitters, parking, and getting to and from Sick Kids. If you stop taking part in our study, we will still pay your for expenses. A certificate of volunteer hours will also be provided to recognize your time.

Participation:

It is your choice to take part in this study. You can stop at any time. The care you get at Sick Kids will not be affected in any way by whether you take part in this study. If you are a Sick Kids employee, this will not impact your employment status. Study results will be accessible to you on request once they become available.

This study has been reviewed and approved by the SickKids and CHEO Research Ethics Boards. These are hospital committees that include people from different professional backgrounds. These Boards review all human research that takes place at their hospitals. Their goal is to ensure the protection of the rights and welfare of people participating in research. The Board’s work is not intended to replace a parent or child’s judgment about what decisions and choices are best for them. For information regarding
patient’s rights in research studies, you may contact the Research Ethics Manager at Sickkids (416-813-5718) or the Chair of the Research Ethics Board at CHEO (613 737-7600 extension 3272). This person cannot provide any health-related information about the study, please contact the study investigators for this information.

Compensation:

During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you any of this money now or in the future because you took part in this study. If you become ill or are harmed because of study participation, we will treat you for free. Your signing this consent form does not interfere with your legal rights in any way. The study staff, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

Conflict of Interest:

Dr. O’Connor, and the other research team members, have no conflict of interest to declare

Sponsorship

This study is funded by the Canadian Institutes of Health Research and sponsored by Dr. O’Connor and the SickKids Department of Physiology and Experimental Medicine.

Consent:

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right not to take part in the study and the right to stop at any time. My decision about taking part in the study will not affect my health care at Sick Kids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about who I am will be given to anyone or be published without first asking my permission.
7) I have read and understood pages 1 to 3 of this consent form. I agree, or consent, to take part in this study.

_________________________________________________________ ________________________________
Printed Name of Participant & Age Participant’s signature & date
_________________________  ____________
Printed Name of person who explained consent  Signature & date

_________________________  ____________
Printed Witness’ name  Witness’ signature & date

(only needed if participant does not read English)

If you have any questions about this study, please call any of the investigators:

Deborah L. O’Connor PhD, RD (416) 813-7844; Mark Tremblay, PhD (613) 737-7600 ext. 4114; Lise Dubois, PhD, RD (613) 562-5398; Cynthia Colapinto PhD(candidate), RD 613-737-7600 ext. 4191

For day to day study issues call: Research Associate (Susanne Aufreiter) Telephone (416 813 5894)

If you have questions about your rights as a participant in a study or for information on whom to contact in the event of injuries during a study, please call the SickKids Research Ethics Manager at 416-813-5718 or the CHEO Research Ethics Manager at (613) 737-7600 extension 3272.
Appendix E

Questionnaire: Examining the folate status of Canadians

Date: __________________________

Assigned ID Number: _____________________________

General Socio-demographic Questions

1. Age: __________________________

2. Sex:
   i. Male
   ii. Female

3. [Were/Was] [you/your child] born a Canadian citizen?
   iii. Yes (Go to Qu.5)
   iv. No (Go to Qu. 4)
   v. DK, RF (Go to Qu. 4)

4. How many years have [you/he/she] lived in Canada?
   i. Enter number of years: _____________________
   ii. DK, RF (Go to Qu.5)

5. People living in Canada come from many different cultural and racial backgrounds. [Are/Is] [you/your child]: (Read categories to respondent. Mark all that apply)
   i. White?
   ii. Aboriginal (specify North American Indian, Métis or Inuit?)
   iii. Chinese?
   iv. South Asian (e.g., East Indian, Pakistani, Sri Lankan)?
   v. Black?
   vi. Filipino?
   vii. Latin American?
viii. Southeast Asian (e.g., Cambodian, Indonesian, Laotian, Vietnamese)?
ix. Arab?
x. West Asian (e.g., Afghan, Iranian)?
xi. Japanese?
xii. Korean?
xiii. Other – Specify _____________________________
6. What is your income level?
   i. Less than 25,000
   ii. 25,000 - 64999
   iii. 65,000 and higher

7. What is the highest degree, certificate or diploma you have obtained?
   i. No post-secondary degree, certificate or diploma
   ii. Trade certificate or diploma from a vocational school or apprenticeship training
   iii. Non-university certificate or diploma from a community college, CEGEP, school of nursing, etc
   iv. University certificate below bachelor’s level
   v. Bachelor’s degree
   vi. University degree or certificate above bachelor’s degree

8. Are you currently attending a school, college or university?
   i. Yes
   ii. No
   iii. Don’t know, refused

Questions related to folate intake (If under 14 years of age, select N/A for Qu. 6 to 8)

9. Do you smoke?
   i. Yes
   ii. No
   iii. Not applicable

10. How often do you have an alcoholic drink?
    i. I don’t drink
11. Do you use birth control or contraceptives? *(If male select N/A)*
   i. Yes
   ii. No
   iii. Not applicable

If so what kind? ____________________________________________________________

12. Are you currently on any medications?
   i. Yes
   ii. No

If so what medications are you on? List below:
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

13. Do you have a medical history that includes any of the following conditions:
   i. A history or presence of any clinically sign gastrointestinal pathology (such as chronic diarrhea, IBD, partial gastrectomy) Yes/No
ii. Any unresolved GI symptoms (diarrhea/vomiting) Yes/No

iii. Any steatorrhea or other conditions that interfere with absorption, distribution, metabolism or excretion of folic acid. Yes/No

Are you pregnant? (If male and/or under 14 years of age select N/A)

a. Yes
b. No
c. Not applicable

Dietary Intake of Folate

15. How often do you eat hot or fortified cold breakfast cereals? (for example: twice a day, three times a week, once a month?)
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

16. How often do you eat white bread, including bagels, rolls, bread, tortillas?
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

17. How often do you eat any kind of pasta, (including spaghetti, noodles, pasta salad or macaroni and cheese)?
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

18. How often do you usually eat: lettuce or green leafy salad with or without other vegetables?
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

19. How often do you usually eat: spinach, or greens like chard, collards and mustard greens?
20. How often do you eat: all other types of vegetables, excluding those already mentioned?
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

21. How often do you eat: orange juice or oranges?
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

22. How often do you usually eat: beans like pinto, red, black or white beans, or refried (included as part of a burrito)?
   i. Per day
   ii. Per week
   iii. Per month
   iv. Per year

23. Have you taken a folic acid containing supplement(s) in the past 30 days?
   a. Yes
   b. No
24. a. If so, what brand(s) and dose(s) do you take? Brand: _______________ (list if more than one taken)
   Dose: _______________

24. b. If so, how often do you take your folic acid containing supplements?
   i. Everyday
   ii. Every second day
   iii. 1-2 per week
   iv. less than 1 per week

25. Do you use any meal replacement/power bars or shakes?
   i. Yes
   ii. No

26. Why would you/do you use folic acid containing supplements?
   i. Not eat right
   ii. Make sure get enough of a vitamin(s)
   iii. Believe it makes you healthier
   iv. Good for helping baby grow
   v. Doctor recommended it
   vi. Other reason ________________________________

27. If you don’t take your folic acid containing supplements every day, what is the reason?
   i. Forget
   ii. Nausea or vomiting-morning sickness-from supplement
   iii. Pill too big
   iv. Don’t like take supplements
   v. Not important
   vi. Other reason ________________________________

28. Do you take a B12 containing supplement?
   i. Yes
   ii. No

29. a. Are you aware of how much folic acid you are supposed to take:
   i. Yes
29. b. How much? __________________

30. a. Are you aware when you should start taking folic acid for pregnancy?

   i. Yes
   ii. No

30. b. When should you start? _________________

31. Who is the person who gave you advice about when to start taking folic acid and how much to take?

   i. Doctor
   ii. Dietitian
   iii. Friend
   iv. Midwife
   v. Consult group (ie Motherrisk)
   vi. Media (please specify)
   vii. Other (please specify)