Maternal one-carbon nutrient status and effects on DNA methylation and hydroxymethylation in newborn infants

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Nutritional Sciences
University of Toronto

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Abstract

DNA methylation is an important epigenetic determinant in gene expression, aberrancies of which are mechanistically related to the development of several diseases. During embryogenesis, a new DNA methylation pattern of the fetus is established, rendering this period highly susceptible to environmental modifiers. Maternal dietary intake and status of one-carbon nutrients (folate, vitamins B₆, B₁₂, choline, and betaine) have the potential to modulate DNA methylation via the provision of S-adenosylmethionine. The primary objective of this study was to determine the effects of maternal one-carbon nutrient status on DNA methylation and hydroxymethylation in umbilical cord blood mononuclear cells. This study also characterized folate and pyridoxal 5’ phosphate (vitamin B₆) concentrations in Canadian pregnant women and in umbilical cord blood. Demographic and dietary information was assessed in 368 pregnant women. Maternal blood samples were collected in early pregnancy and at the time of delivery when an umbilical cord blood sample was also collected. Blood concentrations of one-carbon nutrients including serum and RBC folate, serum vitamin B₁₂, plasma pyridoxal 5’ phosphate, plasma choline and betaine were measured. Mononuclear cells from umbilical cord blood were
extracted and total global DNA 5-methylcytosine and 5-hydroxymethylcytosine content was calculated. Folate concentrations in maternal and cord blood were high, while pyridoxal 5’phosphate concentrations were adequate. There were no strong associations between one-carbon nutrient concentrations and DNA methylation or hydroxymethylation, although maternal concentrations measured in early pregnancy seemed to have more association than later time point measurements. Further studies are warranted to elucidate the potential impact of maternal one-carbon nutrient status on DNA methylation and hydroxymethylation in the developing fetus, which has the potential to modulate disease risk, including cancer, osteoporosis, metabolic and cardiovascular diseases, in the offspring later in life.
Acknowledgements

I would like to extend my sincerest gratitude to the extraordinary people who made this thesis possible.

First, I would like to thank my supervisor, Dr. Young-In Kim, for your guidance and support throughout this research project. I have learned so much from our conversations and I greatly appreciate your time and effort to make this project successful. I especially appreciate your patience and understanding over the years. Thank you for everything.

Thank you also to my co-supervisor, Dr. Debbie O’Connor, not only for your academic guidance but also for your wonderful advice outside of academia. I appreciate our conversations discussing how to balance work and life, something you are an expert at.

Thank you to my academic committee member, Dr. Howard Berger. Your clinical expertise and advice has been invaluable to the success of this project. I have greatly enjoyed working with you.

A heartfelt thank you to everyone who contributed to the work presented in this thesis. Ruth Croxford - a huge thank you for your amazing statistical advice. You have a remarkable gift of making very complicated topics easy to understand. Dr. Susanne Aufreiter and Aneta Plaga – thank you for contributing to the blood analyses and for your support at the O’Connor research lab. Kyoung-Jin Sohn - thank you for your assistance with experiments and study logistics, especially when I was on maternity leave. I would also like to extend my gratitude to those involved in other analyses for this thesis: Dr. Sang-Woon Choi, Dr. Stephanie A. Tammen, Dr. Marie A. Caudill, Olga Malysheva, Dr. Yvonne Lamers, and Theresa Schroeder. Finally, I would like to thank the staff at St. Michael’s Hospital in the Women’s Health Clinic, the offices of Drs. Tessler, Steele and Mocarski, Labour and Delivery, and core laboratory facilities, especially Chris Edgar and Jessica Upward, for implementing and supporting our research objectives.
A special thanks to Anna Ly, Shannon Masih and Carly Visentin. Anna - I wouldn’t have survived without you! You are one of the main reasons our study was so successful. Shannon - you kept me going when my confidence was drained. Thank you for all your support. Carly - thank you for holding down the fort while I was on maternity leave. It was a pleasure working with you during your final year.

Thank you to the past and current members of the Kim lab: Dr. Sung-Eun Kim, Christine Nash, Julie Crowell, Lisa Ishiguro, Michael Yang, Shaidah Deghan, Joanna Warzyszynska, Denise Kim, Heajin Cheon, Eszter Pigott, David Im, and Anne Fard and undergraduate students Nicole Jain, Andrew Wong and Ashley Moon - you all were a pleasure to work with.

To the core Running Rogues Bibiana García-Bailo, Joanne Brathwaite and Matt Parrott – thank you for making me laugh through the tough times.

To my family - thank you Mom, Dad, and Doug for your love and support throughout this process. I appreciate all the Skype conversations, which helped keep me grounded when I needed it the most.

Finally, to my husband Pete. Thank you for your love and support and all the sacrifices you made to help me complete this degree. I am so grateful to have such a loving husband and father to our son Luke. I am looking forward to the fourth addition to the family.
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List of Abbreviations

4-PA, 4-pyridoxic acid

5-methylTHF, 5-methyltetrahydrofolate

AI, Adequate Intake

ALL, acute lymphoblastic leukemia

ALT, alanine aminotransferase

BHMT, betaine homocysteine methyltransferase

CBC, complete blood count

CβS, cystathionine beta synthase

CCHS, Canadian Community Health Survey

CHDH, choline dehydrogenase

CI, confidence interval

CpG, cytosine-guanine dinucleotide

CUBN, cubilin

DFE, dietary folate equivalent

DHFR, dihydrofolate reductase

DMG, dimethylglycine

DMR, differentially methylated region

DOHaD, Developmental Origins of Health and Disease

DRI, dietary reference intake

EAR, Estimated Average Requirement

FA, folic acid

FFQ, food frequency questionnaire
FR, folate receptor
FUT2, α(1,2)-fucosyltransferase
Hcy, total homocysteine
HC, haptocorrin
HDAC, histone deacetylase
holoTC, holotranscobalamin
IF, intrinsic factor
IGF2, insulin-like growth factor 2
IPS, integrated prenatal screening
LC-MS, liquid chromatography-mass spectrometry
LINE-1, long interspersed nucleotide element-1
MAT, methionine adenosyltransferase
MBD, methyl-CpG binding protein
MMA, methylmalonic acid
MMAA, methylmalonic aciduria type A protein
MNC, mononuclear cell
MoBa, The Norwegian Mother and Child Cohort Study
MRP, multidrug resistance-associated proteins
MS, methionine synthase
MTHFD, methylenetetrahydrofolate dehydrogenase
MTHFR, methylenetetrahydrofolate reductase
MTR, 5-Methyltetrahydrofolate-homocysteine methyltransferase
MTRR, methionine synthase reductase
MUT, methylmalonyl-CoA mutase
NEST, The Newborn Epigenetic STudy
NHANES, National Health and Nutrition Examination Survey
NORCCAP, The Norwegian Colorectal Cancer Prevention screening study
NTD, neural tube defect
OR, odds ratio
PABA, p-aminobenzoic acid
PAR, Predictive Adaptive Response hypothesis
PC, phosphatidylcholine
PCFT, proton coupled folate transporter
PCR, polymerase chain reaction
PE, phosphatidylethanolamine
PEMT, phosphatidylethanolamine N-methyltransferase
PL, pyridoxal
PLP, pyridoxal 5'-phosphate
PM, pyridoxamine
PMP, pyridoxamine 5'-phosphate
PN, pyridoxine
PNP, pyridoxine 5'-phosphate
PREFORM, PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant
RBC, red blood cell
RDA, Recommended Dietary Allowance
RFC, reduced folate carrier
RR, relative risk
SAH, S-adenosylhomocysteine
SAM, S-adenosylmethionine
SMH, St. Michael’s Hospital
SNP, single nucleotide polymorphism
SOGC, Society of Obstetricians and Gynecologists of Canada
TC, transcobalamin
TCblR/CD320, transcobalamin receptor
THF, tetrahydrofolate
TMAO, trimethylamine N-oxide
UMFA, unmetabolized folic acid
UL, tolerable upper limit
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Chapter 1: Introduction

Adequate dietary intakes and blood status of one-carbon nutrients such as folate, vitamins B$_6$ and B$_{12}$, choline and betaine during pregnancy are essential to ensure optimal fetal development and growth due to their essential role in nucleotide biosynthesis and biological methylation reactions (1). In particular, adequate prenatal and periconceptional folate status has been shown to be critical in the prevention of neural tube defects (NTDs) (2). This led to implementation of mandatory folic acid fortification in many countries, including Canada and the United States (US) in 1998. Mandatory folic acid fortification led to lower rates of NTDs in North America and is considered a public health policy success (3, 4). During pregnancy, in addition to consumption of a varied and balanced diet, it is recommended that additional folic acid be consumed, which is generally achieved through folic acid fortified foods and supplementation. In Canada, it is common for prenatal supplements to contain a folic acid dose equivalent to the tolerable upper limit (UL) (i.e., 1000 µg/d). A growing body of evidence, albeit controversial, suggests there may be adverse effects of high levels of folic acid exposure in subgroups of the North American population including pregnant women and their newborn infants (5).

Several human studies have linked high maternal folic acid intake with detrimental outcomes in the offspring including small for gestational age babies (6), asthma and wheezing (7), and obesity and insulin resistance in adolescent children (8); however, not all studies have found a detrimental association (9-14). In contrast, there is a lack of data on maternal vitamin B$_6$ intakes and concentrations of pyridoxal 5’ phosphate (PLP), the active form of vitamin B$_6$ in maternal and cord blood. Several studies have found low maternal PLP concentrations to be associated with adverse pregnancy and birth outcomes (15-17), although the data are inconsistent (18, 19).
Finally, associations between low maternal status of other one-carbon nutrients, vitamin B$_{12}$ and choline, and adverse pregnancy and birth outcomes (20-27) have been identified, as well as health effects in the offspring including increased abdominal adiposity and insulin resistance (8) and delayed cognitive development (28, 29).

One-carbon nutrients, including folate and vitamin B$_6$, are important for biological methylation reactions, specifically in the remethylation of homocysteine pathway. Dietary folate and choline enter the pathway as substrates while vitamin B$_6$ and B$_{12}$ acts as cofactors for enzymes involved in the regeneration of S-adenosylmethionine (SAM), the universal methyl donor for most biological methylation reactions (30, 31). Together, these nutrients have the potential to modulate methylation reactions, including that of DNA. DNA methylation is an important epigenetic modification that plays a pivotal role in gene expression and genomic stability and hence, is critical for cell differentiation and development. A newly emerging epigenetic modification linked to DNA methylation is DNA hydroxymethylation, which has been shown to have independent effects on gene activity. A particularly critical period for the establishment of DNA methylation and hydroxymethylation is during the embryonic stage. During this period, DNA methylation and hydroxymethylation patterns undergo epigenetic programming, which erases the parental patterns and establishes a new pattern that is maintained postnatally. Folate and other one-carbon nutrients have the potential to modulate the methyl donor “pool”, impacting SAM available for DNA methylation reactions and S-adenosylhomocysteine (SAH), which can inhibit DNA methylation reactions. Hence, we chose to investigate whether or not the maternal status of one-carbon nutrients could modulate DNA methylation and hydroxymethylation in cord blood mononuclear cells (MNC) of the newborn infant (Figure 1.1).
Figure 1.1. Conceptual model of thesis. Maternal dietary intake of one-carbon nutrients impacts maternal and fetal blood concentrations that subsequently can have an epigenetic effect in the offspring, specifically affecting DNA methylation and hydroxymethylation content.

Due to the concerns involving high folic acid exposure of the developing fetus including aberrant embryonic development, small for gestational age babies, and risk of asthma and atopic disease (6, 32-35), it is extremely important to identify the status of folate in Canadian pregnant women and their newborn infants. Low vitamin B₆ status in pregnant women has previously been linked to adverse pregnancy and birth outcomes, but the results are equivocal (17, 36-38). Furthermore, there is very little information on the vitamin B₁₂ and choline status in pregnancy and their effects on pregnancy and birth outcomes. Lastly, to identify how these one-carbon nutrients may have a lasting effect on the health and disease susceptibility of the newborn, it is important to determine how folate, vitamin B₆, and other one-carbon nutrients such as vitamin B₁₂, choline, and betaine may affect DNA methylation and hydroxymethylation in the offspring. An emerging body of evidence suggests that aberrancies and dysregulation of epigenetic programming during...
embryogenesis may be a link between the intrauterine fetal environment and disease susceptibility, including cancer, osteoporosis, metabolic and cardiovascular diseases, of the offspring later in life (39-41). Therefore, the research objectives of this thesis were as follows:

1) To determine and characterize maternal and cord blood concentrations of folate and vitamin B₆ in a cohort of Canadian pregnant women and their newborn infants
2) To determine maternal and fetal factors that may influence maternal and cord blood concentrations of folate and vitamin B₆
3) To determine whether or not maternal blood concentrations of folate, vitamin B₆ and B₁₂, choline and betaine during pregnancy affect total global DNA methylation and hydroxymethylation in cord MNCs.

This study was a large prospective observational study, entitled the PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant (PREFORM) Study (ClinicalTrials.gov identifier: NCT02244684). The primary objective of the PREFORM study was to determine the effects of maternal intake of folate and folic acid on DNA methylation of cord blood MNCs. Between November 2010 and January 2012, 368 healthy pregnant women between 12 to 16 weeks gestation were recruited from St. Michael’s Hospital (Toronto, Ontario). Consenting participants completed a Baseline Demographic and Health Questionnaire and a food frequency questionnaire (FFQ) and a maternal blood sample was collected. At the second study visit (between 35 and 37 weeks gestation), participants completed the FFQ again. At the time of delivery, maternal and cord blood samples were collected. Concentrations of serum folate, red blood cell (RBC) folate, plasma unmetabolized folic acid (UMFA), plasma PLP, serum vitamin B₁₂, plasma methylmalonic acid (MMA), plasma total choline, betaine, dimethylglycine (DMG),
and trimethylamine N-oxide (TMAO) were determined in maternal blood at recruitment and at delivery and in cord blood. Finally, DNA from cord blood MNCs was extracted and total global 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) content was measured. Several fetal genetic variants in selected genes involved in one-carbon metabolism were determined to assess their potential modifying effects on DNA methylation and hydroxymethylation in cord blood MNCs.

Overall, this study characterized the folate and vitamin B<sub>6</sub> status of pregnant women in Canada and investigated the effects of maternal folate, vitamins B<sub>6</sub> and B<sub>12</sub>, choline and betaine status on DNA methylation and hydroxymethylation in cord blood MNCs. Due to its comprehensive nature, this study provides valuable insight for future studies aimed at investigating the effects of specific one-carbon nutrients on the health of newborn infants.

This thesis consists of 8 Chapters; Chapter 2 is a comprehensive review of the literature. Chapter 3 outlines the rationale, research questions, hypotheses, and objectives of the study. Chapter 4 describes the overall study design, recruitment methods, characteristics of the study cohort, and my contributions to the PREFORM Study. Chapters 5 and 6 describe maternal and cord blood folate and vitamin B<sub>6</sub> status of our cohort, respectively, and Chapter 7 addresses the primary objective of the PREFORM study, which was to determine the effects of maternal one-carbon nutrient status on DNA methylation and hydroxymethylation in cord blood MNCs. Finally, Chapter 8 summarizes the overall conclusions of the research findings and future directions.
Chapter 2: Literature Review

Modified from:


Ly A*, Hoyt L*, Crowell J, Kim YI. Folate and DNA Methylation. Antioxidant Redox Signaling 17: 302-326, 2012 (doi:10.1089/ars.2012.4554) (*co-first authors; equal contribution; published under maiden name Hoyt)

2.1 Folate

2.1.1 Definition, chemical structure and dietary sources

Folate is the overarching term used to describe naturally occurring dietary folates and synthetically manufactured folic acid (1). The main structure of folate and folic acid consists of a 2-amino-4-hydroxy-pteridine moiety attached to p-aminobenzoic acid (PABA) with one or more glutamate residues attached via γ-peptide bonds (Figure 2.1). Folate metabolism involves acquisition of one-carbon units, which can be oxidized or reduced at the N5 and N10 positions. Dietary folates have reduced pteridine rings and are characterized by their polyglutamylated tails whereas folic acid is monoglutamylated and fully oxidized.
Figure 2.1. Chemical structures of (A) folic acid and (B) folate. The main structure of folate and folic acid is a pterin ring attached to p-aminobenzoic acid (PABA) with one or more glutamate residues attached via γ-peptide bonds. Dietary folates have reduced pteridine rings and are characterized by their polyglutamylated tails (B) whereas folic acid is monoglutamylated and fully oxidized at the N5 and N10 positions (A). Reprinted with permission from Kim 2007 (42).

Although endogenous folate is synthesized by colonic bacteria (43) and can be absorbed across the large intestine (44, 45), mammals cannot synthesize folate and therefore must obtain folate from the diet for its biological functions (46). Main dietary sources of folate include leafy green vegetables, citrus fruits, organ meats, and legumes (1). Synthetic folic acid from supplements and fortified foods also significantly contributes to total folate intake (47). Due to their structural difference, folic acid is more stable and therefore, more bioavailable than naturally occurring
folates (46). Naturally occurring folates rapidly lose their activity in foods as they are easily oxidized in low pH (1). Also, folate bioavailability varies widely depending on the food source and preparation method (1). Approximately 50-75% of naturally occurring folate is lost through food harvesting, storage, processing and preparation (1). In contrast, folic acid is highly stable for months or even years (1).

2.1.2 Absorption and Metabolism

Folate and folic acid are absorbed in the duodenum and jejunum of the small intestine (1) and more recently found to be absorbed across the colon (45, 48). Folates enter the enterocyte as monoglutamates and therefore polyglutamylated folates found in the diet require the action of glutamate carboxypeptidase II (GCPII) to cleave the glutamate chain to one glutamate residue (Figure 2.2). Subsequently, monoglutamyl folate is transported across the proximal small intestine by active transport (1). However, in pharmacological ranges (i.e. > 10 µmol/L), folate is absorbed by passive diffusion in a nonsaturable manner. Due to fortification practices and supplemental doses, these concentrations are achievable and are responsible for the appearance of unmetabolized folic acid (UMFA) found in the circulation (49-54).

Folate is transported across cell membranes via two classes of systems: transmembrane carriers and folate-binding protein-mediated systems. The reduced folate carrier (RFC), proton coupled folate transporter (PCFT), a family of low-affinity membrane carriers (multidrug resistance-associated proteins (MRPs), and mitochondrial and lysosomal folate transporters act as transmembrane carriers of folate (46, 55). RFC and PCFT are the best characterized in intestinal folate absorption. Although RFC and PCFT are both highly expressed in the small intestine, they...
prefer different folate forms and function optimally under different pH conditions, allowing for continuous folate absorption throughout the intestine regardless of form or physiological conditions. As its name suggests, RFC has a high affinity for reduced folates and a low affinity for folic acid. It also functions optimally at physiological pH and is virtually inactive below pH 6.5 (56). On the other hand, PCFT functions best at pH below 6.5 and has negligible activity at a neutral pH (46, 55). PCFT has similar affinity for reduced folates and folic acid, although 5-methyltetrahydrofolate (5-methylTHF) transport is more efficient than folic acid transport at pH 6.5. The MRP family of transporters serves as exporters of folate out of tissues (46, 55). Folate affinity is very low for these transporters but they have a high capacity for folate transport. Less is known about the mitochondrial and lysosomal folate transporters (46). The mitochondrial folate transporter is distinct from other folate transporters in that it only transports reduced folates, not folic acid or anti-folates such as methotrexate. Lysosomal folate transporter has been shown to transport methotrexate polyglutamate into lysosomes (46). However, little else is known about its involvement in folate transport.

There are three high-affinity folate receptors (FRs) (α, β, and γ) that make up the folate binding protein-mediated systems of folate transport (46, 55). Both FR α and β are expressed in fetal tissue and a number of adult tissues, although very low levels are found in the gut mucosa. The FRs have a high affinity for a variety of folate forms and the highest for folic acid. Unlike RFC and PCFT, FRs bind and transport folate across the cell membrane via an endocytotic mechanism which, once in the cell, releases the folate, possibly with the combined action of PCFT. FRs are generally found only on the apical membrane of cells and therefore they are responsible for folate transport into the cell whereas transmembrane carriers, being expressed on
both the apical and basolateral membranes, are responsible for folate transport in both directions (57).

Most folate absorbed by the liver from the portal circulation is found in the form of monoglutamylated 5-methylTHF. After 5-methylTHF is metabolized to polyglutamate derivatives in the liver, it is either retained in the liver for storage or converted back to the monoglutamate form before being released into the blood or bile.

Once monoglutamylated folate enters the cell via one of the aforementioned transport methods, folate is returned to its polyglutamylated form via folylpolyglutamate synthase (FPGS) in order to trap folate in the cell. Polyglutamylated folates are also better substrates for one-carbon metabolic reactions. Also, contributing to intracellular folate homeostasis is the action of γ-glutamyl hydrolase (GGH), which cleaves the terminal glutamate residues of folylpolyglutamates, creating monoglutamyl folate, which can exit the cell via RFC and PCFT. Most folate in portal circulation is found in the form of monoglutamylated 5-methylTHF and is absorbed by the liver.
2.1.3 Biochemical Functions

Folate participates in the transfer of one-carbon units involved in nucleotide biosynthesis, the methionine cycle and biological methylation reactions (46). As an essential cofactor for the de novo biosynthesis of nucleotides (46, 58, 59), folate is important in DNA synthesis, stability and
integrity, and repair. Unlike naturally derived folates, folic acid must first be converted to dihydrofolate (DHF) and then to tetrahydrofolate (THF) by DHF reductase (DHFR) primarily in the liver. Only then can biologically inactive folic acid participate in one-carbon metabolism, although it has been hypothesized UMFA in the circulation may compete with active forms of folate for folate transporters, binding sites, and folate-related enzymes (59-61).

THF is the active form of folate, which participates in one-carbon metabolism through its conversion to other forms of folate necessary for de novo synthesis of nucleotides, remethylation of homocysteine, and generation of SAM (Figure 2.3). SAM is the universal methyl donor for >100 biological methylation reactions (31, 62). 5-MethylTHF is synthesized in a nonreversible reaction from the intracellular coenzymatic form of folate, 5,10-methyleneTHF, by the enzyme methylenetetrahydrofolate reductase (MTHFR) (63). 5,10-MethyleneTHF is the methyl donor for the nonreversible methylation reaction, catalyzed by thymidylate synthase of deoxyuridine-5-monophosphate to deoxythymidine-5-monophosphate (thymidylate), a precursor for DNA synthesis. The synthesis of thymidylate results in the oxidation of 5,10-methyleneTHF to the inactive DHF, which can be converted back to THF by DHFR. 5,10-MethyleneTHF can also be oxidized to 10-formylTHF for de novo purine synthesis. Therefore, 5,10-methyleneTHF is critical in maintaining the balance of the nucleotide pool for DNA synthesis. In the methionine cycle, 5-methylTHF transfers single methyl groups to homocysteine, catalyzed by methionine synthase (MS) and the cofactor vitamin B₁₂ (64, 65), to synthesize methionine. MS is ubiquitous in all tissues, which ensures adequate supply of SAM for biological methylation reactions (64). Methionine is then converted to SAM via methionine adenosyltransferases (MAT1A and MAT2A). Alternatively, the remethylation of homocysteine can occur via folate-independent
pathways utilizing betaine or choline; however, this occurs only in the liver and kidney (66).

After donating the methyl group, 5-methylTHF is converted to THF and then to 5,10-methyleneTHF by serine hydroxymethyltransferase (SHMT), a vitamin B<sub>6</sub> dependent enzyme (67, 68). 5,10-MethyleneTHF is a key substrate in folate metabolism, which can be directed towards nucleotide biosynthesis or towards methionine regeneration (69).

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**Figure 2.3. Simplified scheme of the methylation pathway involving one-carbon nutrients.**

Enzymes are in **bold**. AICAR, aminoimidazole carboxamide ribonucleotide; BHMT, betaine homocysteine methyltransferase; -CH<sub>3</sub>, methyl group; CBS, cystathionine beta synthase; DHFR, dihydrofolate reductase; DMG, dimethylglycine; DNMTs, DNA methyltransferases; FAD, flavin adenine dinucleotide; GAR, glycaminide ribonucleotide; MAT, Methionine adenosyltransferases; Met, methionine; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; PEMT, phosphatidylethanolamine N-methyltransferase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; SAHH, SAH hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate. TS, thymidylate synthase. Modified with permission from Zhao et al. (55).
2.1.4 Folate Intake Requirements

Due to bioavailability differences between folate and folic acid, dietary folate intake is expressed as dietary folate equivalents (DFEs). Radiolabelled isotope studies have shown that about 85% of monoglutamylated folates are absorbed compared to 50% of polyglutamylated folates (70-72). Therefore, the totally dietary folate (naturally occurring folate and folic acid from fortified foods) in DFEs is calculated by the following equation:

\[
\mu g \text{ DFE} = x \mu g \text{ dietary folate} + 1.7 (x \mu g \text{ folic acid})
\]  

Dietary Reference Intakes (DRIs) established by the Institute of Medicine a number of intake reference values used for planning and assessing dietary intakes of healthy populations. Determination of the DRIs for folate is based on observational and experimental studies. The estimated average requirement (EAR) and recommended dietary allowance (RDA) for both males and females 19 years old and above is 320 and 400 µg/d DFEs, respectively (Table 2.1). There is an increased metabolic demand for folate during pregnancy due to the rapid rate of one-carbon transfer reactions including those for DNA synthesis and cell division, and for maternal and fetal tissue growth and development (73). Therefore, during pregnancy, the EAR and RDA of folate (520 µg/d and 600µg/d DFE, respectively) exceed those in the non-reproductive state (320 µg/d and 400µg/d DFE, respectively) (1). The EAR for pregnancy was established from metabolic studies in pregnant women. These studies concluded an additional 200 µg/d DFEs to the non-reproductive EAR (for a total of 520µg/d DFEs) was necessary to maintain adequate RBC folate concentrations in pregnancy. Since approximately half of all pregnancies are
unplanned and the neural tube closes 3-4 weeks after conception, a time when many women are unaware they are pregnant, the Food and Nutrition Board of the US Institute of Medicine recommend women of childbearing age between ages 15 to 45 years consume a diet rich in folate as well as 400 µg folic acid/d from a supplement 2-3 months prior to conception and during pregnancy (1). The Society of Obstetricians and Gynecologists of Canada (SOGC) also recommend 400 µg folic acid/d for women with no known risk of an NTD affected pregnancy yet recommend 4000µg folic acid/d for at least 3 months prior to conception and until 12 weeks gestation for women who have had a previously NTD-affected pregnancy or have a personal history of NTDs (74).

Table 2.1. Dietary Reference Intakes (DRIs) for females at various life stages

<table>
<thead>
<tr>
<th>Age and life stage</th>
<th>EAR (µg/d DFEs)</th>
<th>RDA (µg/d DFEs)</th>
<th>UL (µg/d folic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, 19y+</td>
<td>320</td>
<td>400</td>
<td>1000</td>
</tr>
<tr>
<td>Female, pregnant</td>
<td>520</td>
<td>600</td>
<td>1000</td>
</tr>
<tr>
<td>Female, lactating</td>
<td>450</td>
<td>500</td>
<td>1000</td>
</tr>
</tbody>
</table>

DFE, dietary folate equivalents; EAR, estimated average requirement; RDA, recommended dietary allowance; UL, tolerable upper limit.

The UL for folate pertains to folic acid intake only and is set at 1000 µg/d for adults 19 years and older and pregnant and lactating women. The UL was determined as the folic acid level that may mask the hematological effects of vitamin B₁₂ deficiency and give rise to or exacerbate neurological damage (1). Masking occurs when individuals with vitamin B₁₂ deficiency-related megaloblastic anemia consume folic acid. Folic acid corrects the anemia because it can be
converted to THF and 5,10-methyleneTHF while bypassing the methyl-folate trap (75). The conversion of 5,10-methyleneTHF to 5-methylTHF by MTHFR is irreversible, while vitamin B\textsubscript{12} is necessary for MS activity to convert 5-methylTHF to THF, thus causing a buildup of 5-methylTHF. Furthermore, the regeneration of methionine from homocysteine by MS is diminished, causing a buildup of homocysteine and impairment of the methylation cycle. Therefore, treating the hematological effects with more folic acid will permit DNA biosynthesis but not correct the methylation pathway leading to hypomethylation of proteins involved in myelination (76). Case reports in humans and experimental studies in monkeys demonstrated high folic acid intakes can correct megaloblastic anemia often seen in vitamin B\textsubscript{12} deficiency, but neurological damage can progress (1).

In order to extrapolate animal models to human dietary intakes, estimations of equivalent dietary intakes have been proposed. The National Research Council states the basal dietary requirement for folate for rats and mice is 1-2 mg folic acid/kg diet and 0.5 mg folic acid/kg diet, respectively (77). For rodents, published studies generally use a control diet of 2 mg folic acid/kg diet (78-85). The 2 mg folic acid/kg diet contains approximately 4000 kcal/kg diet, which translates to 0.5-1 mg of folic acid in 2000 kcal. This level of folic acid expressed relative to caloric content approximates the RDA of 400\(\mu\)g DFEs in humans consuming a daily average of 2000 kcal (1). However, due to inherent differences in folate metabolism between human and rats such as increased DHFR activity in the rodent liver (61), production of folate by intestinal bacteria (44) and the fact that rodents ingest their own feces (77), the selected dietary folic acid levels may not accurately reflect the corresponding levels in humans.
2.1.5 Biomarkers of Folate

The measurement of folate in serum or plasma and RBCs are the two most common biomarkers of folate status. Serum folate is indicative of short-term folate intake and can be influenced by recent folate and/or folic acid consumption prior to blood draw. Since RBCs have a lifespan of approximately 120 days, RBC concentrations are indicative of long-term (~3 months) folate intake and it measurement is not influenced by recent folate intake. Therefore, RBC folate measurement is considered the gold standard for determining folate status (86). Plasma homocysteine is functional indicator of folate status; however, it is not a specific marker as homocysteine is also influenced by smoking, blood pressure, renal function, thyroid function, medications, and genetic polymorphisms (e.g. MTHFR polymorphism) as well as status of vitamins B₂, B₆ and B₁₂ (87, 88). Although not a clinical biomarker of folate status, the measurement of UMFA in serum or plasma is becoming increasingly important since the detection of UMFA is considered to reflect high intake levels of folic acid commonly found in fortified countries and/or supplemented populations. UMFA is found to be increasing prevalent in blood samples of many populations, and is not limited to countries with mandatory folic acid fortification (89, 90). Observational studies have found detectable levels of UMFA in children, adolescents, and adults (91, 92), older adults (53), pregnant women and umbilical cord blood (50) and in 4 day old infants (93).

Analysis of folates in serum/plasma, RBCs, and other biological fluids can be accomplished by three methods; microbiological, protein-binding, and chromatographic assays (Table 2.2).
Table 2.2. Comparison of folate measurement assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Assay Principle</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiological</td>
<td>Growth of bacteria ((L.\text{rhamnosus})) is proportional to amount of folate in sample</td>
<td>Very sensitive</td>
<td>Does not distinguish between individual folate forms</td>
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<tr>
<td></td>
<td></td>
<td>Cost effective</td>
<td>Can be affected by antibiotics</td>
</tr>
<tr>
<td>Protein-binding</td>
<td>Folate binding proteins “extract” folate from samples, can be radio- or nonradio-labelled</td>
<td>Quick results</td>
<td>Narrow detection range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High-throughput analysis</td>
<td>Binding proteins respond differently depending on total folate concentration</td>
</tr>
<tr>
<td>Chromatographic</td>
<td>Individual forms of folate are separated and quantified based on their interaction with the adsorbent material. May be quantified by measuring the mass to charge ratio.</td>
<td>Can measure individual folate forms</td>
<td>Extensive sample cleaning</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Expensive specialized equipment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trained personnel</td>
</tr>
</tbody>
</table>

The microbiological assay was developed over 50 years ago for the measurement of total folate concentrations. The principle behind the microbiological assay is that the microorganism \((L.\text{rhamnosus})\), formerly known as \(L.\text{casei}\), cannot grow in a folate-free medium and therefore growth is proportional to the amount of folate present in the sample. \(L.\text{rhamnosus}\) responds differently to various polyglutamyl chain lengths, with a full response from mono-, di-, and triglutamates but a lower response with increasing glutamate residues. Compared with other analytical methods, the microbiologic method is very sensitive and cost effective. Despite its age, the \(L.\text{rhamnosus}\) microbiological assay procedure is still considered the “gold standard” for folate measurement since it remains the simplest and most easily interpretable method for assessment of overall folate status (94). However, it does not distinguish between different forms of folate and only measures total folate.
Protein binding assays were developed as a quick and easy analysis alternative to the lengthier microbiological method. Protein-binding assays use folate binding-proteins (commonly from milk or milk fractions) to “extract” or bind folate from the sample. Initial methods employed radiolabelled folates for detection. However, more recent methods are non-radiolabelled and have been modified for high throughput clinical applications with minimum user involvement. Protein-binding assays also respond differently to folate forms depending on the one-carbon substituent (methyl-, methylene-, etc.) and number of glutamate residues. Polyglutamates generally provide an increased response compared with monoglutamates but this is dependent on the overall folate concentration in the sample. Also, protein-binding assays have a narrow detection range and its dilution linearity may be inaccurate.

Unlike microbiologic and protein-binding assays, chromatographic assays are advantageous because they can separate and measure individual folate forms. However, they require extensive sample clean up and attentiveness due to complex sample manipulation. Higher order methods such as isotope-dilution/liquid chromatography/tandem mass spectrometry have been developed which most closely measures the true value of folate in a sample. Using this method provides a reliable reference method for individual folate forms and has been shown to be in good agreement with the microbiologic assay (95).

The variations in the aforementioned methods of folate analysis make it very difficult, especially with RBC folate concentrations, to compare concentrations across studies and assay methods as it is well known that RBC folate concentrations vary depending on the laboratory in which they are conducted and the assay employed (96-99). For example, RBC folate concentrations measured by the Bio-Rad Quantaphase II protein-binding assay were found to measure 45% lower than the microbiological assay (86). In contrast, two studies have shown RBC folate
concentrations measured by the Immulite 2000 immunoassay were higher than those determined by the microbiologic assay (98, 100). Conversion equations have been calculated for some assay comparisons (98, 101). However, caution should be taken when applying these conversions to data outside the original studies.

The assays used in the current thesis were the Access Folate System for serum folate and the Elecsys Folate Assay for RBC folate, both protein-binding immunoassays. The immunoassays are both high throughput methods commonly used in clinical practice processing a high volume of biological specimens. Although the microbiological assay is considered the gold standard, the immunoassay measurement is widely available in hospital or clinical laboratories and provides an opportunity to directly compare folate values across different laboratories. Furthermore, we were able to measure blood folate concentrations in real time throughout the day by utilizing the laboratory system within the hospital. This prevented samples from being frozen, thawed or stored for periods of time prior to analysis, all issues which can affect the quality of the folate measurement (95). Plasma UMFA was measured using liquid chromatography-mass spectrometry (LC-MS). This method is the only assay that can isolate and quantify individual forms of folate. It is a highly sensitive and specific analytical method which most closely represents the true UMFA value in plasma (95).

Surprisingly, given the lack of consistency among the various folate assays, serum and RBC folate concentration cutoffs for folate deficiency are commonly used and accepted. RBC folate concentration less than 305 nmol/L (140 ng/ml), the level at which hematological changes occur (e.g., macrocytic anemia) and when plasma homocysteine concentrations start to increase (1, 102). A serum folate concentration less than 7 nmol/L (3 ng/ml) is also an indicator of folate
deficiency; however, this is less commonly used since serum folate is very sensitive to recent folate and/or acute alcohol intake (1).

Due to increasing folate status arising from fortification and supplement use, cutoffs for high folate status have been suggested, although not formerly accepted. A serum folate concentration above 45 nmol/L has been used to indicate a high serum folate concentration. This was arbitrarily based on the calibration point in the BioRad immunoassay before a sample needed to be diluted in the 1999-2004 National Health and Nutrition Examination Survey (NHANES) (103). This concentration is coincidently also close to the 95th percentile for the 1988–1994 NHANES data (39 nmol/L) (104). A RBC folate concentration cutoff at or above 1360 nmol/L using Bio-Rad immunoassay was derived from the 1999-2004 NHANES data. This represents the concentration of the 97.5th percentile of folate concentrations in 23,527 males and females, ages 4 and older (103). More recently, three high RBC folate concentrations cutoffs have been proposed (1450, 1800, and 2150 nmol/L) (105). These were estimated from the range of postulated cutoffs previously used in the literature (101, 106, 107). It was reported 16%, 6%, and 2% of the Canadian population surveyed using the Canadian Health Measures Survey (CHMS) had RBC folate concentrations (adjusted from Immulite 2000 immunoassay to microbiologic assay) above 1450, 1800, and 2150 nmol/L, respectively (105).

2.1.6 Folate and Health

Due to its role in purine and thymidylate synthesis and methylation reactions, folate is essential for proper cell division, normal growth and development, and therefore plays a crucial role in human health and disease (46). The health outcomes associated with inadequate and excess
intakes of folate have been summarized in Table 2.3.

Table 2.3. Health outcomes associated with inadequate and excess folate

<table>
<thead>
<tr>
<th>Inadequate folate</th>
<th>Excess folate</th>
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<tbody>
<tr>
<td>Congenital anomalies (e.g., NTDs)</td>
<td>Antifolate drug resistance</td>
</tr>
<tr>
<td>Cognitive impairment</td>
<td>Masking B12 deficiency</td>
</tr>
<tr>
<td>Megaloblastic anemia</td>
<td>Decreased natural killer cell cytotoxicity</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>Cancer progression</td>
</tr>
<tr>
<td>Cancer initiation</td>
<td></td>
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</tbody>
</table>

Except for megaloblastic anemia and NTDs, the precise nature and magnitude of the relationship between folate status and the risk of these diseases have not been uniformly consistent and remain to be clearly elucidated (1, 5, 108). An overwhelming body of evidence supports the link between folate deficiency and the development of megaloblastic anemia and NTDs (1). In contrast, the association between folate deficiency and the risk of cardiovascular disease, other congenital defects, adverse pregnancy outcomes, and cognitive impairments has not been unequivocally established (1). In particular, a large body of epidemiologic evidence has suggested an inverse association between folate deficiency and an increased risk of coronary heart disease and stroke, in part through hyperhomocysteinemia (109-113). However, most of placebo-controlled randomized clinical trials of folic acid supplementation, either alone or in combination with other B vitamins, have reported a null effect for coronary heart disease (114-116). In contrast, folic acid supplementation, alone or in combination with other B vitamins, appears to be beneficial for stroke (116, 117). Several meta-analyses and systemic reviews of
these clinical trials have confirmed folic acid supplementation has no effect on coronary heart disease but may be beneficial for stroke (118-121).

In addition, a substantial amount of epidemiologic evidence suggest an inverse association between folate status (assessed by dietary folate intake or by blood measurements of folate) and the risk of several malignancies including cancer of the lungs, oropharynx, esophagus, stomach, colorectum, pancreas, cervix, ovary, prostate, and breast and the risk of neuroblastoma and leukemia (42, 122-124). The highly controversial relationship between folate and carcinogenesis has been most widely studied for colorectal cancer. In general, the portfolio of epidemiologic and clinical evidence indicates ~20 – 40% reduction in the risk of CRC or its’ well established precursor, adenoma, in subjects with the highest dietary intake or blood levels of folate compared with those with the lowest intake or blood levels (42, 122, 124, 125). The role of folate in colorectal carcinogenesis has been further strengthened by the observations that genetic polymorphisms in the folate metabolic pathway (e.g. MTHFR C677T polymorphism) modify colorectal cancer risk (126-128). Although several small intervention studies have demonstrated that folic acid supplementation can improve or reverse surrogate endpoint biomarkers of colorectal cancer (129-135), placebo-controlled randomized clinical trials have shown that folic acid supplementation can increase (136), decrease (137) or have a null effect on (130, 138, 139) the risk of recurrent adenomas. Of particular interest is the Aspirin/Folate Polyp Prevention Study in which folic acid supplementation was associated with a 67% increased risk of recurrent advanced adenomas with a high malignant potential in individuals with prior colorectal adenomas (136). Furthermore, folic acid supplementation was associated with an increased risk of prostate cancer in this trial (140).
The data from animal studies have suggested a dual effect of folate on the development and progression of colorectal cancer. In normal tissues, folate deficiency predisposes them to neoplastic transformation, and modest levels of folic acid supplementation suppress, whereas excessive supplemental doses enhance the development and progression of (pre)neoplastic lesions (42). In contrast, folate deficiency has an inhibitory effect, whereas folic acid supplementation has a promoting effect, on the progression of established (pre)neoplastic lesions (42). Several biologically plausible mechanisms relating to folate’s role in one-carbon transfer reaction have been proposed to explain this dual role of folate in colorectal cancer development and progression (141).

With increased folate status in many fortified countries, there has been increased interest in the health effects of excess folate. In particular, there is concern that excess folate, especially folic acid, may increase the risk for certain cancers. In a study from Norway in which the primary endpoint was the cardiovascular outcome in response to folic acid and other B vitamin supplementation, treatment with folic acid (800 µg/d) and vitamin B₁₂ (0.4 mg/d) for a median period of 36 months significantly increased overall cancer incidence and mortality by 21% and 38%, respectively (142). However, a meta-analysis of eight randomized trials of folic acid supplementation involving 37,485 individuals at increased risk of cardiovascular disease, folic acid supplementation of a median duration of 5 years had no significant effects on vascular outcomes (the primary endpoint), overall cancer incidence, cancer mortality or all-cause mortality (113). Another meta-analysis reported no overall effect of folic acid supplementation on colorectal cancer risk; however, there was a non-significant trend toward an increased risk of colorectal cancer in the three trials involving patients with previous adenomas and in all 13 trials (studies with previous colorectal adenoma patients or individuals at high risk for cardiovascular
disease) included in the meta-analysis (143). Two ecologic studies that examined a temporal postfortification trend of colorectal cancer incidence in the US, Canada and Chile reported increased colorectal cancer rates in these countries following fortification, suggesting that folic acid fortification may have been wholly or partly responsible for this disturbing trend (144, 145). However, two large prospective epidemiologic studies conducted after folic acid fortification in the US have suggested a colorectal cancer-protective effect of consuming adequate amounts of folate and have not demonstrated a colorectal cancer-promoting effect of folic acid supplementation (146, 147).

Excess folic acid has also been linked to masking B\textsubscript{12} deficiency (75) since high folate can correct the negative hematological effects of vitamin B\textsubscript{12} deficiency but not the neurological effects and therefore, cognitive decline can persist without notice. This can occur when abundant folate is available to maintain purine and thymidine synthesis but in the presence of insufficient vitamin B\textsubscript{12}, 5-methylTHF is unable to donate its methyl group to homocysteine to generate methionine. This is due to suboptimal MS function, a vitamin B\textsubscript{12} dependent enzyme (Figure 2.3). The masking of vitamin B\textsubscript{12} deficiency is of particular concern in the elderly as several studies have found an increased risk of cognitive impairment in older adults with combination high folate and low vitamin B\textsubscript{12} status (148-151).

Another concern linked to excess folate/folic acid is resistance or tolerance to antifolate drugs used against arthritis (152, 153) and cancer (154-156). Antifolate drugs are designed to interfere with enzymes in folate metabolism. Therefore, excess folate and folic acid can compete with these drugs, potentially decreasing the efficacy and potency of the drug.
High folic acid intake, as indicated by UMFA in the circulation, has been associated with decreased natural killer cell cytotoxicity in a group of American postmenopausal women (157). An inverse U-shaped association between combined dietary folate and supplemental folic acid intake and natural killer cell cytotoxicity was reported, while increased plasma UMFA concentrations were associated with decreased natural killer cell cytotoxicity in women over the age of 60 y (157).

Overall, both folate adequacy and excess can have effects on human health and disease. In particular, an emerging body of evidence has raised concerns that high folate status, especially from folic acid supplementation, may have adverse health effects in humans.

2.1.7 Folic Acid Fortification

The beneficial effect of optimal folate status during childbearing age and pregnancy on NTDs risk is well established. The Medical Research Council Vitamin Study Research Group conducted a large (n=1195) multicenter randomized controlled double-blinded study across seven countries to investigate the effect of periconceptional folic acid supplementation on NTD risk in women with a previous NTD-affected pregnancy. In this trial, a 72% protective effect of 4000 µg of folic acid was observed in women at risk of having an NTD-affected pregnancy. Two additional studies reported that a dose of 800µg/d (158) and 400µg/d (159) was also protective against NTDs.

Based on the overwhelming body of evidence showing a protective effect of periconceptional folic acid supplementation on NTD risk (2, 158-160), the Canadian and US governments
mandated folic acid fortification in 1998 (4, 161). The addition of 150µg FA/100g white wheat flour and 200µg folic acid/100g cornmeal and enriched pasta (140µg FA/100g for all three grains in the US) led to an approximately 50% decrease in NTD prevalence in Canada and the US, making folic acid fortification a public health policy success (3, 162-164).

Post folic acid fortification, significant increases in North American total folate intakes and serum and RBC folate concentrations (163, 165) were observed. The fortification dose was designed to add an additional daily intake of 100-200 µg folic acid. However, some studies have suggested this is an underestimation (166) and over-fortification may have resulted in folic acid intake significantly greater than what was anticipated (165).

Increases in serum and RBC folate concentrations from pre- to post-fortification are highlighted by the longitudinal data from NHANES. Using pre- (1988-1994) and post- (1999-2004) fortification data, Pfeiffer et al. found serum and RBC folate concentrations increased by 119–161% and 44–64%, respectively, in each age group after fortification. Furthermore, the prevalence of low serum (<6.8 nmol/L) and RBC folate (<305 nmol/L) concentrations declined in women of childbearing age from pre to post fortification era (from 21% to <1% and from 38% to 5%, respectively). Overall, there was a 60% increase in average RBC folate concentrations: from 398 to 636 nmol/L in pre- and post-fortification, respectively (103).

Data representative of 96.3% of the Canadian population from 2007-2009 found virtually no folate deficiency in the Canadian population post-fortification as determined by RBC folate concentrations (<305 nmol/L). In the same survey, 40% of the Canadian population had concentrations above 1360 nmol/L, the high cutoff adopted from the 97.5th percentile of
NHANES 1999-2004 data (107). Similarly, recent data from the Center for Disease Control and Prevention’s (CDC) Second Nutrition Report found less than 1% of the US population is folate deficient using NHANES data from 2003-2006 (167).

Although folate intakes and concentrations have substantially increased since folic acid fortification, there are still segments of the North American population with mandatory fortification that might be susceptible to suboptimal RBC folate concentrations including women of childbearing age. A RBC folate concentration of at least 906 nmol/L was established as the level of maximal protection against having a NTD-affected pregnancy (168). This cutoff was established from an Irish case-control study involving over 56,000 pregnant women. The study found a dose-response relationship between RBC folate concentrations and NTD risk and concluded there was an eight-fold decrease in NTD risk with an RBC folate concentration of 906 nmol/L compared to 305 nmol/L (168). Another optimal NTD risk cutoff (~1000 nmol/L) using two large studies in China (total n=249,045) has recently been proposed (169). However, the former cutoff is more conventionally used. In the US, >90% of women of childbearing age (20-59 years old) had RBC folate concentrations <906 nmol/L pre-fortification (170). Surprisingly, this percentage did not decrease post-fortification, although median concentrations increased from 505 nmol/L to 587 nmol/L (170). In Canada, 22% of women of childbearing age (15 to 45 years old) had RBC folate concentrations below 906 nmol/L (107). It was determined folic acid supplement use was the biggest predictor of reaching optimal RBC folate concentrations above the NTD-protection level (171). These facts underscore the importance of folic acid fortification and the need for folic acid supplementation before and during pregnancy for NTD protection. However, the dose of periconceptional folic acid supplementation and the fortification level of folic acid remain controversial issues.
2.1.8 Folate in Pregnancy

2.1.8.1 Role of Folate in Pregnancy Outcomes

The demand for folate is high for the developing fetus and therefore, circulating folate concentrations are higher in cord blood compared to maternal blood (1, 172, 173). Inadequate maternal folate intakes can have detrimental effects on pregnancy and birth outcomes and offspring’s health status, given folate’s role in optimal cellular growth in the placenta and fetus. Indeed, adverse pregnancy outcomes including preterm birth, low birth weight, and intrauterine growth restriction have been reported to be associated, albeit inconsistently and equivocally, with suboptimal maternal folate status (19, 174, 175). For example, higher plasma folate concentrations at ~27 weeks of gestation were associated with a longer gestation (0.12 wk per SD increase in folate; 95% CI: 0.02, 0.21) and there was a negative trend with the risk of all preterm birth (delivery at <37 wk of gestation; odds ratio [OR]: 0.79; 95% CI: 0.63, 1.00) (19). Supplementation with a multivitamin containing folic acid prior to 16 weeks gestation decreased the risk of preeclampsia by 43% in an American pregnant population, although specific micronutrient(s) responsible for this decrease was not specified (176). Similarly, supplementation with a multivitamin containing folic acid decreased the risk of preeclampsia by 63% in a Canadian pregnant population (177). However not all studies have found a relationship between folic acid and preeclampsia risk (178, 179).

As previously mentioned, the relationship between inadequate maternal folate intake and NTDs is well established. In addition, the majority of studies have shown an inverse association between maternal folic acid intake and congenital heart defects. For example, the Hungarian
intervention trial has shown an inverse association between periconceptional folic acid-containing multivitamin supplementation and risk of congenital heart defects (180). Similarly, observational studies investigating folic acid-containing multivitamin supplementation or folic acid supplementation alone in the US (181, 182), the Netherlands (183) and China (184) have confirmed these findings. Finally, a recent population-based case-control study found a reduced risk of congenital heart defects in mothers who supplemented with folic acid (average dose ~6 mg/d) during the critical period of development relating to the particular congenital heart defect (185). The relationship between maternal folate status and the risk of other congenital defects (e.g., cleft palate), however, has not been well established and is tenuous at best (186). The observed inconsistencies regarding the effects of maternal folate status on pregnancy outcomes could be related to the overall folate status of the population studied (187).

2.1.8.2 Folate Exposure in Pregnancy

Studies from Canada and the US have found the majority of women take supplements containing folic acid prior to and during pregnancy (188-191). Most prenatal vitamins available on the market contain 400-1000 µg folic acid. Branum et al. found 78% of American women reported taking a folic acid- or iron-containing multivitamin at least 30 days prior to pregnancy (191). Supplement use in the first, second and third trimesters was ~55%, ~77%, and 89%, respectively, in this study (191). Hoyo et al. found 12% of American women had daily folic acid intakes above the UL of 1000 µg folic acid/d before and during pregnancy (190). Sherwood et al. found in a group of Canadian pregnant women (n=61) from Southern Ontario, approximately 84% of the women supplemented at 36 weeks gestation, with 1000 µg/d or greater of folic acid. From food record analysis, mean dietary folate intake of these women during pregnancy was reported at 562 ± 106 µg/d DFEs per day. However, when folic acid supplement use was accounted for,
67% of pregnant women exceeded the UL (188). The median (95% CI) dietary folate intake during pregnancy in a larger Canadian sample (n=599) from Alberta increased from 269 (240, 303 \(\mu g/d\)) to 306 (290, 318 \(\mu g/d\)) from the first to third trimester (192). Intakes at these levels are considered inadequate; however, conversion to DFEs to account for the higher bioavailability of folic acid was not done. Nonetheless, nearly all subjects were supplementing with folic acid of at least 600 \(\mu g/d\) during pregnancy, indicating adequate intakes (192).

In the last decade and a half, there have been a number of studies investigating maternal blood levels of folate during pregnancy (8, 50, 89, 172, 173, 191-195) (Table 2.4). Two studies from mandatory folic acid fortified North American populations are described herein. A study in the US characterized blood concentrations across all three trimesters of pregnancy in 1296 women from the 1999 to 2006 NHANES data (191). As previously mentioned, the majority of women took folic acid supplements prior to and during pregnancy. This was reflected in high median (95% CI) RBC folate concentrations that increased incrementally across the gestational periods (the first (1255 nmol/L [1048, 1525 nmol/L]) second (1527 nmol/L [1449, 1630 nmol/L]) and third (1773 nmol/L [1694, 2012 nmol/L]) trimesters). In the Alberta Pregnancy Outcomes and Nutrition (APrON) study (192), nearly all 599 of the subjects reported taking at least 600 \(\mu g/d\) folic acid during pregnancy. Similar to the American data, RBC folate concentrations were high across the gestational periods (median (95%CI) in first (1280 nmol/L [1114, 1393 nmol/L]), second (1504 nmol/L [1450, 1568 nmol/L]), and third (1462 nmol/L [1421, 1529 nmol/L]) trimesters], although concentrations seem to plateau after the second trimester (192). Overall, these findings reveal that folic acid supplementation practices in North America pregnant women are widespread and result in elevated RBC folate concentrations during pregnancy.
Table 2.4. Summary of studies investigating maternal folate and homocysteine concentrations

<table>
<thead>
<tr>
<th>Location</th>
<th>FA fortification?</th>
<th>FA supplementation?</th>
<th>Serum/RBC folate method</th>
<th>UMFA method</th>
<th>Sample size</th>
<th>Time of blood draw</th>
<th>Fasted samples?</th>
<th>Serum folate (nmol/L) median(range)</th>
<th>RBC folate (nmol/L) median(range)</th>
<th>UMFA (nmol/L) median(range)</th>
<th>% with detectable UMFA</th>
<th>Hcy (µmol/L) median(range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Voluntary</td>
<td>Not addressed</td>
<td>immuno</td>
<td>MBA</td>
<td>69</td>
<td>childbirth</td>
<td>No</td>
<td>13 (7-34)</td>
<td>580 (252-1635)</td>
<td>-</td>
<td>-</td>
<td>7.1 (3.3-21.0)</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes (17%)</td>
<td>MBA</td>
<td>HPLC</td>
<td>201</td>
<td>childbirth</td>
<td>Yes</td>
<td>26 (14-41)</td>
<td>1627 (1044-2093)</td>
<td>0 (0-1.34)</td>
<td>-</td>
<td>8.3±2.9</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>No</td>
<td>immuno</td>
<td>LC-MS</td>
<td>82</td>
<td>34-36 wks</td>
<td>No</td>
<td>27±20</td>
<td>813±475</td>
<td>-</td>
<td>-</td>
<td>5.6±1.6</td>
</tr>
<tr>
<td>Japan</td>
<td>No</td>
<td>No</td>
<td>immuno</td>
<td>LC-MS</td>
<td>82</td>
<td>28 wks</td>
<td>No</td>
<td>23±49</td>
<td>961 (736-1269)</td>
<td>0.29</td>
<td>-</td>
<td>5.9±1.4</td>
</tr>
<tr>
<td>India</td>
<td>Voluntary</td>
<td>No</td>
<td>immuno</td>
<td>Bio-Rad adjusted for MBA</td>
<td>562</td>
<td>childbirth</td>
<td>Yes</td>
<td>21 (7-70)</td>
<td>945 (278-2180)</td>
<td>-</td>
<td>-</td>
<td>0.15</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes (29%)</td>
<td>immuno</td>
<td>HPLC</td>
<td>20</td>
<td>childbirth</td>
<td>Yes</td>
<td>19 (6-47)</td>
<td>1185 (702)</td>
<td>-</td>
<td>-</td>
<td>90%</td>
</tr>
<tr>
<td>Germany</td>
<td>Limited</td>
<td>Yes (84%)</td>
<td>MBA</td>
<td>-</td>
<td>87</td>
<td>childbirth</td>
<td>Yes</td>
<td>26 (20)</td>
<td>-</td>
<td>-</td>
<td>44%</td>
<td>5 (4.3-8.2)</td>
</tr>
<tr>
<td>USA</td>
<td>Voluntary</td>
<td>Yes (60-89%)</td>
<td>RBC-MBA</td>
<td>-</td>
<td>138</td>
<td>36 wks</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5 (2.3)</td>
</tr>
<tr>
<td>Australia</td>
<td>Voluntary</td>
<td>Yes (nearly all taking ≥600 µg/d)</td>
<td>-</td>
<td>-</td>
<td>1296</td>
<td>1, 2, 3 trimester</td>
<td>Yes?</td>
<td>-</td>
<td>1st: 1255 (1048, 1525), 2nd: 1527 (1449, 1630), 3rd: 1773 (1694, 2012)</td>
<td>122-520, varies by trimester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Mandatory</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>122</td>
<td>1, 2, 3 trimester</td>
<td>Yes</td>
<td>36 (35, 36) in 1st and 2nd tri</td>
<td>1st: 1280 (1114, 1393), 2nd: 1504 (1450, 1568), 3rd: 1462 (1421, 1529)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Guerra-Shinohara et al. 2002* 
*Molloy et al. 2002* 
*Obeid et al. 2005* 
*Takimoto et al. 2007* 
*Yajnik et al. 2008* 
*Sweeney et al. 2009* 
*Obeid et al. 2010* 
*Hure et al. 2011* 
*Branum et al. 2013* 
*Fayyaz et al. 2014* 

*aMolloy et al. reported median(IQR) unless otherwise stated. bObeid et al. 2005 reported geometric means±SD. cTakimoto et al. reported mean±SD. dYajnik et al. reported median(25th-75th percentile). eObeid et al. reported median(10th-90th percentile). fHure et al. reported median(IQR). gFayyaz et al. reported median(95%CI). Immuno, protein-binding immunoassay; MBA, microbiological assay; Hcy, homocysteine; LC-MS, liquid chromatography-mass spectrometry*
Fewer studies have investigated folate concentrations in cord blood (Table 2.5). All studies investigating maternal and cord folate concentrations found higher concentrations in cord blood compared to maternal (50, 172, 173, 193). Three studies investigating UMFA reported detectable levels in cord plasma in 55-100% of samples (50, 89, 93). Interestingly, this occurred even in populations without mandatory folic acid fortification and no prenatal supplementation (89, 93).

Table 2.5. Summary of studies investigating cord folate and homocysteine concentrations

<table>
<thead>
<tr>
<th>Location</th>
<th>FA fortification?</th>
<th>FA supplementation?</th>
<th>Sample size</th>
<th>Serum/RBC folate method</th>
<th>UMFA method</th>
<th>Serum folate (nmol/L)</th>
<th>RBC folate (nmol/L)</th>
<th>UMFA (nmol/L)</th>
<th>% with detectable UMFA</th>
<th>Hcy (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Voluntary</td>
<td>Not addressed</td>
<td>69</td>
<td>Immunoassay</td>
<td>-</td>
<td>28</td>
<td>1027</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes (17%)</td>
<td>201</td>
<td>MBA</td>
<td>-</td>
<td>47</td>
<td>2142</td>
<td>-</td>
<td>-</td>
<td>7.9±2.9</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>No</td>
<td>82</td>
<td>Immunoassay</td>
<td>HPLC/MB</td>
<td>61±21</td>
<td>-</td>
<td>0.42</td>
<td>100%</td>
<td>5.9±1.9</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes</td>
<td>9</td>
<td>-</td>
<td>HPLC</td>
<td>-</td>
<td>-</td>
<td>0.28</td>
<td>85%</td>
<td>5.4±1.9</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>No</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.21</td>
<td>-</td>
<td>10.8±3.8</td>
</tr>
<tr>
<td>UK</td>
<td>Yes</td>
<td>Yes (29%)</td>
<td>23</td>
<td>Immunoassay</td>
<td>LC-MS</td>
<td>15.8±3.5</td>
<td>-</td>
<td>(0-0.60)</td>
<td>55%</td>
<td>5.3</td>
</tr>
<tr>
<td>Germany</td>
<td>Limited</td>
<td></td>
<td>29</td>
<td>LC-MS</td>
<td>-</td>
<td>40</td>
<td>(9-79)</td>
<td>(0-0.51)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aMolloy et al. reported median(IQR) unless otherwise stated. bObeid et al. 2005 reported geometric mean±SD. cSweeney et al. 2005 reported mean values. dFryer et al. reported mean±SD. eObeid et al. reported median(10th-90th percentile). MBA: microbiological assay, HPLC: high performance liquid chromatography, LC-MS: liquid chromatography-mass spectrometry.
2.1.8.3 Maternal Folate status and Health Outcomes in the Offspring

In the last decade, research focus has shifted from the effects of low maternal folate intake to those of excessive intakes on the health of the offspring. This is largely due to the drastically increased intakes and blood levels of folate and folic acid resulting from mandatory folic acid fortification in many countries and the prevalent periconceptional use of folic acid supplements. The emerging body of evidence has demonstrated that increased folate intakes during pregnancy may have the potential to modulate disease risks in the offspring (Table 2.6).

High maternal folic acid is generally thought to be protective against pediatric cancers. A nationwide Australian case control study found use of folic acid during pregnancy, but not vitamin B$_6$ or B$_{12}$, was associated with decreased risk of childhood brain tumors (196). Combined data from 12 case control studies in the Childhood Leukemia International Consortium found maternal folic acid supplementation at any period (preconception, pregnancy, trimester) to be protective against leukemia (197). Although a meta-analysis could not attribute the effect to a certain constituent or constituents of multivitamins, it was able to conclude prenatal multivitamins (including folic acid) were protective against leukemia, pediatric brain tumors, and neuroblastoma (198). However, some studies have shown an increased risk of pediatric cancers with prenatal folic acid supplementation (199, 200). Animal studies have shown that maternal folic acid supplementation decreases the risk of colon cancer (80) while it increases the risk of mammary tumors (79) in the offspring.

Data from the Rhea Study in Crete, Greece has reported multiple benefits of high folic acid supplementation (5000 µg/d) in early to mid pregnancy. Babies born to mothers who supplemented with 5000 µg folic acid/d had a 31% decrease in the risk of preterm birth (relative
risk [RR], 0.69; 95 % CI, 0.44, 0.99), a 60% decrease in the risk of a low birth weight newborn (RR, 0.40; 95 % CI, 0.21, 0.76), and a 66% decrease in the risk of delivering a small-for-gestational age (SGA) newborn (RR, 0.34; 95 % CI, 0.16, 0.73) (9). The Rhea Study also found high folic acid supplementation to be associated with improved vocabulary development, communication skills, and verbal comprehension in the children at 18 months old (201). Other studies have found maternal folic acid to be beneficial for language development. Data from the Norwegian Mother and Child Cohort Study (MoBa) reported a significant decline in the incidence of severe language delay in 3 year old children born to mothers who used folic acid-containing supplements from four weeks pre conception to eight weeks gestation compared to those children whose mothers did not (12). A retrospective analysis of the MoBa data found a significant protective effect of maternal folic acid supplementation (four weeks preconception to 8 weeks gestation) on autistic spectrum disorders in children compared to those children who were not exposed to periconceptional or prenatal folic acid (11). This was corroborated by the data from the Generation R Study in The Netherlands, which demonstrated maternal plasma folate status at 13 weeks gestation was not associated with autistic traits in offspring at six years of age, yet preconception use of folic acid was associated with less risk of autistic traits in offspring (202).

The effect of prenatal folic acid supplement use on respiratory health and allergic disease in the offspring is equivocal (203). In an Australian population, folic acid supplementation late in pregnancy (30-34 weeks gestation) has been associated with childhood asthma at 3.5 years of age (7). An increased risk of atopic dermatitis in offspring was reported with serum folate >16.2 nmol/L and serum vitamin B₁₂ >178 pmol/L in children up to two years of age. However, no
association was found between maternal folate status and wheezing or shortness of breath in offspring (33). Two meta-analyses have reported conflicting conclusions: a meta-analysis of 14 studies (10 cohort, 3 nested case-control, and 1 case-control) found no association between childhood asthma and maternal folic acid supplementation (14), whereas a meta-analysis of 26 studies (16 cohort, 7 case-control, and 3 cross-sectional) found maternal folic acid supplementation during early pregnancy may increase the risk of wheeze in early childhood and that offspring homozygous for the MTHFR 677C>T polymorphism would be at high risk of asthma development (204). Further studies are needed to determine the role of prenatal folate intake in the development of respiratory diseases.

Other adverse outcomes in the offspring associated with excessive maternal folic acid supplementation include increased risk of oral clefts (205) and having a small-for-gestational age for height neonate (206). Furthermore, the combination of high maternal folate and low vitamin B_{12} status has been associated with delivering a small for gestational age baby (6) and an increased risk of insulin resistance and obesity in children at six years of age (8).
Table 2.6. The beneficial and adverse effects of periconceptional and prenatal folic acid supplementation on pregnancy and birth outcomes in offspring’s health risk

<table>
<thead>
<tr>
<th></th>
<th>Protective effects</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy outcomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Convincing</td>
<td></td>
<td>• NTDs</td>
</tr>
<tr>
<td>Probable</td>
<td></td>
<td>• Congenital heart defects</td>
</tr>
<tr>
<td>Possible/Insufficient</td>
<td></td>
<td>• Preterm birth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Low birth weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Small for gestational age babies (with low maternal vitamin B&lt;sub&gt;12&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Birth outcomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Convincing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probable</td>
<td></td>
<td>• Pediatric cancers</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Language development</td>
</tr>
<tr>
<td>Possible/Insufficient</td>
<td></td>
<td>• Autistic traits</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Asthma/wheeze in offspring</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Adolescent adiposity and insulin resistance</td>
</tr>
</tbody>
</table>

Overall, these studies indicate high maternal folic acid consumption (through dietary and supplemental sources) and folate status may be associated with both beneficial and adverse effects on health and disease of the offspring. More research is needed to understand the balance between the benefits and risk of increased folic acid exposure in utero.

2.2 Vitamin B<sub>12</sub>

2.2.1 Definition, chemical structure and dietary sources

Vitamin B<sub>12</sub> (cobalamin) is an essential water-soluble B vitamin, which is required for the production of RBCs and for optimal neurological function. Vitamin B<sub>12</sub> is a complex vitamin compound, which is found in many different forms, all of which have the commonality of a
central cobalt atom (Figure 2.4). Depending on the compounds attached to the cobalt atom, different types of cobalamins are formed such as methylcobalamin, cyanocobalamin, hydroxycobalamin, aquacobalamin, and 5'-deoxyadenosylcobalamin. The most stable form of this vitamin is cyanocobalamin, the pharmacological form (1), which is readily converted to the coenzyme forms, methylcobalamin and 5'-deoxyadenosylcobalamin (207). These coenzyme forms are biologically active in human metabolism.

Figure 2.4. Structural formula of vitamin B₁₂ and partial structures of vitamin B₁₂ compounds. The partial structures of vitamin B₁₂ compounds show only those portions of the molecule that differ from vitamin B₁₂. 1: 59-deoxyadenosylcobalamin; 2, methylcobalamin; 3, hydroxocobalamin; 4, sulfocobalamin; 5, cyanocobalamin or vitamin B₁₂. Reprinted with permission from Watanabe 2007 (207).

Vitamin B₁₂ is naturally found in foods of animal origin such as fish, shellfish, chicken, red meat, liver and dairy products. The bioavailability of natural dietary B₁₂ is approximately 50% of synthetic (crystalline) B₁₂ (207).
2.2.2 Absorption and Metabolism

In the healthy digestive system, vitamin B$_{12}$ is first released from food by acid and pepsin in the stomach (1). The free B$_{12}$ attaches to R proteins produced by the salivary glands in the mouth, called haptocorrins, which help transport the B$_{12}$ to the small intestine. Once in the small intestine, pancreatic proteases cleave the haptocorrin-B$_{12}$ and release vitamin B$_{12}$ to be bound with intrinsic factor (IF), a glycoprotein secreted by the parietal cells of the stomach. The IF-vitamin B$_{12}$ complex attaches to specific receptors, cubilin, cubam and amnionless, in the mucosa of the ileum. Once the complex enters the enterocyte, IF dissociates from vitamin B$_{12}$ and is then bound to the plasma binding protein transcobalamin when the vitamin B$_{12}$ is released into circulation. Circulating vitamin B$_{12}$ is always bound to one of two plasma binding proteins: haptocorrin (HC) or transcobalamin (TC). HC binds ~80% of the circulating vitamin B$_{12}$ and acts as a circulating reserve of vitamin B$_{12}$; TC delivers vitamin B$_{12}$ to the tissues through the transcobalamin receptor (TCblR/CD320) (208). Of the two B$_{12}$ transport proteins (HC and TC), B$_{12}$ bound to transcobalamin (called holoTC) represents the fraction of circulating B$_{12}$ available for cellular uptake (209). Healthy adults, with normal gastrointestinal function, are believed to absorb 50% of dietary vitamin B$_{12}$ (207). With age, however, malabsorption of food-bound vitamin B$_{12}$ increases due to gastrointestinal impairments, such as reduced gastric acidity secondary to atrophy, which results in a higher prevalence of vitamin B$_{12}$ deficiency in the elderly (1, 106).

2.2.3 Biochemical Functions
The methylcobalamin form of vitamin B₁₂ serves as one of the key enzymatic cofactors in one-carbon metabolism (1). Vitamin B₁₂ functions as the coenzyme for MS, which catalyzes the transfer of a methyl group from 5-methylTHF to homocysteine, forming methylcobalamin and regenerating THF. In this biochemical pathway, homocysteine is remethylated to methionine in what is considered to be the folate-dependent remethylation pathway of one-carbon metabolism (210) (Figure 2.5). The other metabolically active form of vitamin B₁₂, 5'-deoxyadenosylcobalamin, serves as the coenzyme for L-methylmalonyl-CoA mutase, the enzyme that catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA, an enzymatic reaction which is involved in amino acid and fatty acid metabolism (1, 207) (Figure 2.6).

**Figure 2.5. Biochemical functions of vitamin B₁₂ in one-carbon metabolism.** Enzymes are shown in **bold.** B₁₂, vitamin B₁₂ (methylcobalamin); BHMT, betaine-homocysteine methyltransferase; DMG, dimethylglycine; Met, methionine; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; tHcy, homocysteine; THF, tetrahydrofolate. Figure reprinted with permission from Masih, 2013 (211).
2.2.4 Vitamin B$_{12}$ Intake Requirements

The EAR and RDA for healthy adults 19-50 years of age is 2.0 and 2.4 $\mu$g/d, respectively. The EAR was set as the intake needed to maintain serum vitamin B$_{12}$ concentrations and to prevent the negative hematological effects of vitamin B$_{12}$ deficiency. Since there is no reliable standard deviation for the EAR value, the RDA is set as 120% of the EAR.

According to 2003-2006 NHANES data, adult women consume [median (IQR)] 3.4 (2.5, 5.9) $\mu$g/d from a diet containing enriched cereal grains (212). When ready-to-eat cereal and supplemental sources were included, the median (IQR) intake increased to 16.5 (9.8, 37.0) $\mu$g/d (212). Data from Canada reported estimated intakes, which were less than adequate (intakes less than the EAR) for up to 20% of females $\geq$14 y with diet only; however, when supplements were accounted for inadequacy dropped to <5% (213).

During pregnancy, there is evidence to suggest maternal vitamin B$_{12}$ absorption is more efficient than the non-pregnant state (1). Furthermore, fetal deposition of vitamin B$_{12}$ has been estimated.
to be 0.1 to 0.2 µg/d. Therefore, the EAR and RDA during pregnancy is increased to 2.2 and 2.6 µg/d, respectively (1).

2.2.5 Biomarkers of vitamin B\textsubscript{12}

Vitamin B\textsubscript{12} status can be measured directly in the blood or determined by measuring functional or metabolic biomarkers. Serum B\textsubscript{12} and plasma holotranscobalamin (holoTC) both measure circulating concentrations of B\textsubscript{12}. HoloTC is the active portion of total circulating vitamin B\textsubscript{12}. A serum vitamin B\textsubscript{12} concentration of 150 pmol/L is approximately 3 SDs from the reference range and is a commonly used deficiency cutoff (209). A vitamin B\textsubscript{12} concentration between 150 and 258 pmol/L is often considered marginal or “subclinical” vitamin B\textsubscript{12} deficiency (214, 215). A level of 220 pmol/L is a commonly used cutoff for maximal protection again a NTD during pregnancy (216). Methylmalonic acid (MMA) and homocysteine, both inverse functional indicators, increase when there is an inadequate concentration of vitamin B\textsubscript{12} (Figures 2.5 and 2.6). MMA increases when there is a decrease in the activity of the enzyme L-methylmalonyl-CoA mutase due to an inadequate source of the coenzyme form of vitamin B\textsubscript{12}, 5\textsuperscript{'}-deoxyadenosylcobalamin. There are a number of increased MMA cutoffs indicating vitamin B\textsubscript{12} deficiency, however the most commonly used one is MMA >271 nmol/L (217). Homocysteine also serves as a functional indicator of B\textsubscript{12} status but is considered a non-specific inverse indicator considering that increased levels of homocysteine are also associated with inadequate folate concentrations and to lesser extent, inadequate vitamin B\textsubscript{6} and riboflavin (vitamin B\textsubscript{2}). Given each biomarker’s sensitivity and specificity, a roundtable summary of the NHANES reported that the most definitive assessment of B\textsubscript{12} status requires the measurement of a
combination of at least one biomarker of circulating vitamin B$_{12}$ (serum vitamin B$_{12}$ or holoTC) and one functional biomarker (MMA or homocysteine) (209) (Table 2.7).

Table 2.7. Comparison of vitamin B$_{12}$ status biomarkers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Type of biomarker</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma/serum vitamin B$_{12}$</td>
<td>Circulating measure</td>
<td>Measures total circulating vitamin B$_{12}$ (bound to HC and TC)</td>
<td>Needs to be confirmed with functional biomarker (MMA or homocysteine)</td>
</tr>
<tr>
<td>holo transcobalamin (holoTC)</td>
<td>Circulating measure</td>
<td>Measures active portion of vitamin B$<em>{12}$ (B$</em>{12}$ bound to TC)</td>
<td>holoTC concentrations are understudied</td>
</tr>
<tr>
<td>Methylmalonic acid (MMA)</td>
<td>Functional marker</td>
<td>Elevated MMA (&gt;271 nmol/L) could indicate vitamin B$_{12}$ deficiency</td>
<td>MMA not a good functional marker of B$_{12}$ status in certain cases (e.g. pregnancy)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>More specific to vitamin B$_{12}$ status than homocysteine</td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>Functional marker</td>
<td>Elevated homocysteine (&gt;13 µmol/L) could indicate vitamin B$_{12}$ deficiency</td>
<td>Influenced by other B vitamins (folate, vitamins B$<em>{2}$ and B$</em>{6}$)</td>
</tr>
</tbody>
</table>

2.2.6 Vitamin B$_{12}$ and Health

The metabolic pathways of vitamin B$_{12}$ and folate are integrally related, thereby leading to a correlation between folate and vitamin B$_{12}$ status. High folic acid intake has the potential to mask the hematological symptoms and signs associated with vitamin B$_{12}$ deficiency, considering that both these vitamins play a role in normal blood cell formation. However, folic acid cannot correct vitamin B$_{12}$ deficiency-associated neurological symptoms. Therefore, as mentioned previously, when initial hematological symptoms are masked through the corrective action of
folic acid, the untreated vitamin B\textsubscript{12} deficiency can lead to severe neurological symptoms such as irreversible sensory neuropathy (1).

The main clinical manifestations of vitamin B\textsubscript{12} deficiency are megaloblastic anemia, myelopathy, neurodegeneration, depression and cognitive decline (1, 106, 218).

2.2.7 Vitamin B\textsubscript{12} in Pregnancy

2.2.7.1 Role of vitamin B\textsubscript{12} in Pregnancy Outcomes

Vitamin B\textsubscript{12} is essential for fetal development as it contributes to methyl transfer reactions. As mentioned previously, vitamin B\textsubscript{12} functions as a cofactor for MS, which catalyzes the remethylation of homocysteine to methionine which in turn ensures the provision of SAM, the primary circulating methyl donor necessary for many biological reactions including DNA methylation.

Inadequate B\textsubscript{12} status during pregnancy has been shown to be an independent risk factor for NTDs, which may be in part due to its close metabolic relationship to folate (216, 219). Several case control studies have reported OR between a 2.5 to 5.4 of having a NTD affected pregnancy with low vitamin B\textsubscript{12} status (20, 24, 216, 220-222); however, not all have found an association (223, 224). Molloy et al. have reported a 2 to 3 fold increase OR for NTDs in women in the lowest serum vitamin B\textsubscript{12} quartile (<185 pmol/L) compared to women in the highest quartile (216). This study further suggested vitamin B\textsubscript{12} concentrations should be at least 221 pmol/L prior to pregnancy to reduce the risk of NTDs.
Maternal vitamin B\textsubscript{12} status also appears to influence birth weight. In a study from Bangalore, low maternal serum vitamin B\textsubscript{12} in first and third trimesters as well as in cord blood was significantly associated with lower birth weight in the infants (225). Muthayya \textit{et al.} also reported that low maternal serum vitamin B\textsubscript{12} concentrations during each trimester of pregnancy increased the risk of intrauterine growth restriction (22). However, in Western populations from the Netherlands (226) and Australia (227), adverse pregnancy outcomes such as premature birth, small for gestation age at birth and preeclampsia (226) and preeclampsia and intrauterine growth restriction (227) were not found to be associated with serum vitamin B\textsubscript{12} concentrations during pregnancy.

2.2.7.2 Vitamin B\textsubscript{12} Exposure in Pregnancy

Vitamin B\textsubscript{12} concentrations have previously been shown to decrease throughout pregnancy (192, 228). Reasons for this decline are not limited to hemodilution but also include a decrease in vitamin B\textsubscript{12} absorption, hormonal changes, alterations in vitamin B\textsubscript{12} binding proteins activity or variations in placenta vitamin B\textsubscript{12} transport (1, 229). Increasing nutrient demands of the fetus have also been suggested to lower maternal concentrations (228). The fetus has a high demand for vitamin B\textsubscript{12} for the synthesis of nucleic acids, proteins and methyl groups that are essential for embryogenesis, fetal development and epigenetic programming (195). Over the course of pregnancy, maternal vitamin B\textsubscript{12} is concentrated in the placenta and is subsequently transferred to the fetus. Newborn vitamin B\textsubscript{12} concentrations are twice as high compared to the mother’s and is tightly regulated by maternal status, thereby signifying the importance of maternal vitamin B\textsubscript{12} status for ensuring a sufficient supply to the fetus (229). For example, Obeid \textit{et al.} reported higher cord serum vitamin B\textsubscript{12} concentrations [mean (95% CI): 268 (88, 1018) pmol/L] compared to maternal concentrations [188 (81, 770) pmol/L] (229) and Hure \textit{et al.} reported
higher vitamin B<sub>12</sub> in infants 27 weeks postpartum [median (IQR): 266 (223) pmol/L] compared to maternal concentrations at 20 weeks gestation [177 (91) pmol/L] and 36 weeks gestation [149 (56) pmol/L] (195).

Suboptimal vitamin B<sub>12</sub> status is not uncommon in pregnancy. A cross-sectional study in Canadian women estimated about 1 in every 20 women is at risk for vitamin B<sub>12</sub> deficiency (<125 pmol/L) in early pregnancy (219). An American cross-sectional study reported in pregnant women receiving a daily prenatal supplement containing 12 µg vitamin B<sub>12</sub>, 2, 10 and 13% of the women had deficient blood levels of vitamin B<sub>12</sub> at each trimester, although the vitamin B<sub>12</sub> deficiency cutoff was not stated (228). Despite low levels of vitamin B<sub>12</sub> and a number of other vitamins (thiamin, niacin, vitamin B<sub>6</sub>, vitamin A) in this study, low birth weight, eclampsia, fetal stress, neonatal mortality or morbidity, or abnormal Apgar scores were not associated with these deficiencies (228). A recent study in Colombian pregnant women reported a prevalence of vitamin B<sub>12</sub> deficiency (<148 pmol/L) and marginal deficiency (≥148 and <222 pmol/L) to be 19% (95% CI, 17%, 22%) and 41% (95% CI, 38%, 44%), respectively (230). The APrON study reported pregnant women in the first (n=123) and second (n=521) trimesters had median (95% CI) holoTC concentrations of 92 (84, 100) and 83 (80, 85) pmol/L, respectively (192). HoloTC concentrations were within the normal range (35 to 140 pmol/L) in 88%–91% of the women, depending on the trimester (192). Although this functional biomarker indicates the majority of pregnant women were not vitamin B<sub>12</sub> deficient, the study did not measure an additional biomarker of vitamin B<sub>12</sub> status (serum vitamin B<sub>12</sub>, plasma MMA or plasma homocysteine) which has been recommended to identify subclinical deficiencies (209). In this cohort, the median (95% CI) dietary vitamin B<sub>12</sub> intakes reported at each trimester [3.7 (3.3, 4.0) µg/d, 4.0 (3.7, 4.2) µg/d, and 4.1 (3.8, 4.4) µg/d in the first, second and third trimesters,
respectively] indicate intakes well above the RDA (2.6 µg/d) at each trimester, which may account for the observed low prevalence of vitamin B₁₂ deficiency as suggested by holoTC concentrations (192).

2.2.7.3 Maternal vitamin B₁₂ status and Health Outcomes in the Offspring

Several detrimental outcomes for the offspring have been linked to suboptimal maternal vitamin B₁₂ status. For example, the Pune Maternal Nutrition Study (8, 231, 232) examined the effects of maternal vitamin B₁₂ status on health outcomes in the offspring, given the high prevalence of a vegetarian lifestyle low in vitamin B₁₂ in India. Maternal vitamin B₁₂ concentrations were positively associated with mental and social development quotients in children at two years of age (232). Furthermore, at six years of age, the offspring of the mothers with low vitamin B₁₂ status (<150 pmol/L) at 18 weeks gestation exhibited a much higher level of central adiposity and insulin resistance (measured by HOMA-IR) (8). The highest insulin resistance was observed in children whose mothers had the lowest plasma vitamin B₁₂ and highest RBC folate concentrations (8). Finally, South Asian mothers with vitamin B₁₂ concentrations in the lowest decile (<77 pmol/L) at 28 wk of gestation had children who performed more slowly on sustained attention and short-term memory tests at nine years of age than those born to mothers with vitamin B₁₂ concentrations in the highest decile (>224 pmol/L) (231). This trend in the offspring’s cognitive decline with low maternal vitamin B₁₂ status has been supported by other studies (233-235).
2.3 Vitamin B₆

2.3.1 Definition, chemical structure, and dietary sources

Vitamin B₆ is a water-soluble B vitamin comprised of several vitamers: pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM). These three compounds also exist in the 5’ phosphate forms (PLP, PNP and PMP) (Figure 2.7).

![Figure 2.7. Vitamer forms of vitamin B₆. Reprinted from Gandhi et al. 2012 (Open-access use) (236).](image-url)
The B₆ vitamers act as coenzymes in over 100 biological reactions involving metabolism of carbohydrates, lipids and proteins (1). Rich sources of vitamin B₆ include fish, beef liver, chickpeas, and non-citrus fruits. However, the US population consumes vitamin B₆ primarily from fortified cereals, beef, poultry and starchy vegetables (1).

2.3.2 Absorption and Metabolism

The most abundant form of vitamin B₆ in the human body is PLP followed by PMP. All forms of vitamin B₆ are absorbed in the jejunum. Phosphorylated forms of the vitamin (PLP, PMP, PNP) are dephosphorylated by phosphatase-mediated hydrolysis. This is followed by transport of the nonphosphorylated forms (PL, PM, PN) into the mucosa by nonsaturable passive diffusion (1). The liver receives the majority of the absorbed nonphosphorylated forms of vitamin B₆. PN, PL, and PM are converted back to PNP, PLP, and PMP, respectively, by the action of pyridoxal kinase. Furthermore, PNP and PMP are oxidized to PLP by PNP oxidase. PLP can also be converted to PMP by aminotransferase reactions. PLP is protected from dephosphorylation by phosphatases because it remains bound to various proteins in tissues. When the protein binding capacity for PLP is surpassed, unbound PLP is rapidly hydrolyzed to PL and the nonphosphorylated forms of vitamin B₆ are released by the liver and other tissues into circulation. The liver has the ability to oxidize and release PLP in the excretory form of 4-pyridoxic acid (4-PA), while muscle, plasma and hemoglobin have the highest PLP-protein binding capacities thereby allowing accumulation of very high levels of PLP even when other tissues are saturated (1).
2.3.3 Biochemical Functions

As mentioned previously, the B₆ vitamers act as coenzymes in over 100 biological reactions involving metabolism of carbohydrates, lipids and proteins (1). With respect to one-carbon metabolism, vitamin B₆ is related to folate, vitamin B₁₂ and choline within the numerous pathways involved in the regeneration of SAM from homocysteine. Homocysteine can be cleared via three pathways: remethylation to methionine by MS or betaine-homocysteine methyltransferase (BHMT) in the transmethylation pathway (in the liver and kidney only (66)) or condensation with serine to form the amino acid cysteine in the two-step transsulfuration pathway which requires the biologically active form of vitamin B₆, PLP, as a cofactor. Vitamin B₆ is also involved in the conversion of serine to glycine by acting as a coenzyme for SHMT, which catalyzes the conversion of THF to 5,10-methyleneTHF (Figure 2.3).

2.3.4 Vitamin B₆ Intake Requirements

When establishing the EAR for vitamin B₆, a PLP concentration of at least 20 nmol/L was the major indicator of adequacy, which is reflective of tissue stores. Controlled metabolic studies investigating the effects of a combination diet on plasma PLP concentrations, with the knowledge that approximately 75% of vitamin B₆ from a mixed diet is bioavailable, the EAR for adults aged 19 to 50 years was established at 1.1 mg/d. The RDA was then extrapolated to be 1.3 mg/d, which is approximately 120% of the EAR.

Concentrations of plasma PLP decrease throughout pregnancy, with the most significant drop in the third trimester (1). Hemodilution does not seem to solely explain the drop in PLP
concentrations since the observed 10 nmol/L decrease in plasma PLP in pregnancy is more substantial than what an increase in plasma volume would explain (1). Considering the increased metabolic needs of the mother and developing fetus, and assuming approximately 75% bioavailability of vitamin B₆ from a mixed diet, an estimated increase of 0.5 mg/day was suggested (1). This established the EAR as 1.6 mg/d and the RDA as 1.9 mg/d of vitamin B₆ in pregnancy. Because vitamin B₆ needs are greater in late gestation, 0.5 mg/day of vitamin B₆ may overestimate the additional need in early gestation, but a conservative approach was taken to ensure sufficiency and add 0.5 mg/day to the EAR for non-pregnant women throughout pregnancy (1).

High intakes of vitamin B₆ from food sources have not been associated with adverse effects. However, supplemental doses of pyridoxine (PN), which are used for therapeutic reasons, have demonstrated adverse side effects such as sensory neuropathy and dermatological lesions (1). Therefore, the UL, established for PN, is 100 mg/d in adults. This is based on studies that have linked very high doses of PN with sensory neuropathy (1).

2.3.5 Biomarkers of vitamin B₆

Vitamin B₆ status is most commonly measured by assessing biologically active PLP in plasma since it most closely reflects tissue stores and is indicative of long term status (1). However, vitamin B₆ status can also be measured in RBCs or urine by measuring other individual B₆ vitamers or total vitamin B₆ forms. The major metabolic product of vitamin B₆ is 4-PA (1). Its measurement in plasma has been suggested as a short-term indicator of status (237). However, reference cutoffs currently only exist for PLP. A PLP cutoff of ≥30 nmol/L has been suggested
as adequate B₆ concentration in adults (1). However, several studies involving various populations reported that although a substantial proportion of individuals had plasma PLP concentrations below 30 nmol/L, no corroborating clinical or other data indicating B₆ deficiency was evident in these individuals (1). A PLP concentration above 20 nmol/L in plasma has also been suggested as a cutoff for adequate B₆ concentrations in adults (1). This more conservative cutoff is not accompanied by observable health risks but allows a safety margin to protect against the development of deficiency symptoms (1). This cutoff was also selected as the basis for the vitamin B₆ EAR for adults (1).

Quantification of plasma PLP can be determined by microbiological assays, enzymatic assays, liquid chromatography with fluorescence detection, LC-MS/MS, and most recently, the nonradioactive single-enzyme assay (238) (Table 2.8).
Table 2.8. Comparison of vitamin B<sub>6</sub> measurement assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Assay Principle</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiological</td>
<td>Growth of bacteria is proportional to amount of vitamin B&lt;sub&gt;6&lt;/sub&gt; in sample</td>
<td>Very sensitive</td>
<td>Least selective</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cost effective</td>
<td>Can be affected by antibiotics</td>
</tr>
<tr>
<td>Enzymatic, radioactive</td>
<td>PLP-dependent apo-tirosine decarboxylase converts [1-&lt;sup&gt;14&lt;/sup&gt;C]tyrosine to &lt;sup&gt;14&lt;/sup&gt;CO&lt;sub&gt;2&lt;/sub&gt;, which is trapped and quantified</td>
<td>Quick results</td>
<td>Limited linear range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High-throughput analysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ready to use kits available</td>
<td></td>
</tr>
</tbody>
</table>
| Enzymatic, non-radioactive   | **Reaction 1:** Apo-enzyme recombinant homocysteinase, combines with cofactor PLP to form α-ketobutyrate, H<sub>2</sub>S, and NH<sub>3</sub>.  
**Reaction 2:** H<sub>2</sub>S combines with N,N-dibutylphenylenediamine to form 3,7-Bis(dibutylamino)-phenothiazine-5'-ium chloride. The absorbance of this compound is read at 675 nm. | Non-radioactive                 | Low precision and accuracy                      |
|                              |                                                                                  | Requires small volume of sample |                                                  |
| Liquid chromatographic       | Individual forms of B<sub>6</sub> are separated and quantified based on their interaction with the adsorbent material. May be quantified by measuring the mass to charge ratio. | Can measure individual vitamin B<sub>6</sub> forms | Extensive sample cleaning                       |
| (HPLC, LC-MS/MS)             |                                                                                  | High accuracy and precision    | Trained personnel required                      |
Microbiological assays can be used to determine total vitamin B₆ concentrations in plasma or RBCs. However, they cannot distinguish between different B₆ vitamers and therefore, a measure of only PLP is not possible (238).

Enzymatic assays rely on a PLP dependent enzymatic reaction to quantify PLP. The common enzymatic reaction in radioactive labeled assays is the conversion of [1-¹⁴C]tyrosine to ¹⁴CO₂, by the PLP-dependent apo-tyrosine decarboxylase. The labeled carbon dioxide is trapped and quantified for PLP determination (238, 239). In the non-radiolabelled assay, Apo-enzyme recombinant homocysteinase, combines with cofactor PLP to form α-ketobutyrate, H₂S, and NH₃. In the second reaction, H₂S combines with N,N-dibutylphenylenediamine to form 3,7-Bis(dibutylamino)-phenothiazine-5’-ium chloride. The absorbance of this compound is read for PLP quantification (239). Although enzymatic assays are simpler and quicker than the microbiological assay, there is a small linear range of detection and precision and accuracy, especially with the nonradioactive assay, is questionable (238, 240).

Finally, chromatographic assays, including HPLC and LC-MS/MS, which utilize a mobile phase to selectively quantify individual forms of vitamin B₆, has been shown to be highly specific and accurate. However, time- and cost-intensive measures are needed to maintain satisfactory laboratory procedures with these methods (238).

Comparing eight laboratories utilizing HPLC methods with two utilizing enzymatic assays, Rybak et al. reported imprecision was generally higher with enzymatic assays than with HPLC methods (240). The authors concluded that the two largest issues laboratories face is quality
control and calibration accuracy (240). Overall, similar to folate assays, methodological differences and inter-laboratory variation can lead to difficulties comparing concentrations across populations (240).

The assay used to measure plasma PLP in the current thesis was the enzymatic, non-radioactive assay (239). This method was chosen because a measure of plasma PLP only was desired, and the microbiological assay cannot make the distinction between all vitamin B₆ vitamers. Furthermore, the enzymatic, nonradioactive assay was previously utilized to measure plasma PLP concentrations in over 6000 participants including women in reproductive age in the 2003-2004 NHANES survey cycle (241) and we wished to compare plasma PLP values from the PREFORM study to those of the 2003-2004 NHANES survey.

2.3.6 Vitamin B₆ and Health

Deficiency of vitamin B₆ is rarely seen in the general population due to the vitamin being available in a wide variety of foods, although certain subgroups such as those with gastrointestinal disorders, renal dysfunction, autoimmune diseases or alcohol dependence may be at higher risk of B₆ inadequacy (1). Vitamin B₆ deficiency can lead to microcytic anemia, irregularities in electroencephalogram readings, depression and confusion, and compromised immune function (1). Deficiency symptoms include cracked corners of the mouth and swollen tongue (1).

Vitamin B₆ status has been linked to a number of diseases, most notably coronary heart disease, stroke, and cognitive dysfunction (1). More recently, vitamin B₆ status has been inversely associated with inflammatory diseases, such as rheumatoid arthritis (242) and inflammatory
bowel disease (243), possibly due to increased vitamin B₆ catabolism during systemic inflammation (244), as well as sickle cell anemia (245) and diabetes (246). Although theoretically plausible due to the homocysteine-lowering effect of vitamin B₆, randomized control trials and combined analyses have failed to show vitamin B₆ supplementation, alone or in combination with other B vitamins, reduce the risk of coronary heart disease and stroke (114, 247, 248). With regards to cognitive function, low vitamin B₆ status has been hypothesized to lead to cognitive decline in the elderly (1). However, a systematic review and a Cochrane review concluded there is not enough evidence to support the purported beneficial effect of vitamin B₆ supplementation on cognitive function in the elderly (249, 250).

There is also a considerable body of literature on vitamin B₆ and cancer risk; however, its effect appears to be tissue specific. Emerging evidence suggests a potential association between vitamin B₆ and colorectal cancer in humans. The majority of case-control studies have concluded that high dietary vitamin B₆ intakes are modestly protective against colorectal cancer compared to the low intakes (251), although not all have found a protective effect (252, 253). Nested case-control studies have suggested that plasma PLP is associated with an approximately 30-50% reduction in colorectal cancer risk when comparing the highest to lowest concentrations (251). However, one case-control study has found that plasma PLP concentrations had no effect on the development of colorectal adenomas (254). Large population-based prospective studies, including The Women’s Health Initiative Study (255), the European Prospective Investigation into Cancer and Nutrition Study (256), Physician’s Health Study (257), and Multiethnic Cohort Study (258), have generally found a protective effect of vitamin B₆ status on colorectal cancer risk. However, the Iowa Women’s Health Study (253, 259) found no effect while the Netherlands Cohort Study found an increased risk for rectal cancer in women (260). A meta-
analysis including aforementioned studies reported a lower colorectal cancer risk associated with the highest PLP compared to the lowest PLP concentrations but only a non-significant protective trend for dietary vitamin B₆ intakes (261).

Inconsistencies exist in the case-control studies investigating the relationship between vitamin B₆ and breast cancer risk. Some studies have reported a significant decrease in the risk associated with higher dietary intakes (262), higher circulating PLP concentrations (263), and higher dietary intakes combined with lower alcohol intakes (264), while other studies have reported a lack of association between higher vitamin B₆ intakes and breast cancer risk (265, 266). The majority of prospective studies examining the risk of breast cancer in relation to dietary intakes of vitamin B₆ demonstrated a lack of effect (267-270).

Vitamin B₆ has not been demonstrated to have an effect on prostate cancer risk with respect to dietary and/or supplemental intakes in either case-control (271-276) or prospective (277) studies. However, one prospective study found a significant protective effect of dietary vitamin B₆ intakes on localized prostate cancer (278). Less evidence is available regarding the effects of vitamin B₆ on other cancers.

2.3.7 Vitamin B₆ in Pregnancy

2.3.7.1 Role of vitamin B₆ in Pregnancy Outcomes

A small number of studies have linked low vitamin B₆ status with adverse pregnancy outcomes. In a prospective observational study in China, women with plasma PLP < 30 nmol/L had a decreased probability of conception and an increased risk of early pregnancy loss (17). Using the
same study population, the risk of preterm birth was significantly lower in mothers with plasma PLP $\geq$30 nmol/L preconception (16). In a multi-ethnic population in Singapore, vitamin B$_6$ status [median (IQR) 62 (26–113) nmol/L plasma PLP concentrations at ~27 weeks gestation] was not associated with preterm birth or delivering a small-for-gestational-age neonate (19). A non-randomized experimental study where mothers received 0, 1, 2, or 3 mg pyridoxine hydrochloride during pregnancy (average length of exposure: 7 months) found mothers with plasma PLP $\geq$ 40 nmol/L at the time of delivery delivered heavier neonates than mothers with PLP < 40 nmol/L (37). However, no differences were seen in birth length, head or chest circumference with differing maternal plasma PLP concentrations (37). As plasma PLP concentrations decrease during pregnancy (1), it has not been unequivocally established whether inadequate PLP at the onset of pregnancy or anytime during pregnancy is of clinical importance.

### 2.3.7.2 Vitamin B$_6$ Exposure in Pregnancy

Vitamin B$_6$ status during pregnancy varies by geographic region, possibly due to differing dietary patterns, supplement use, and fortification practices (Table 2.9). For example, in a group of Japanese women not taking supplements and with considerably low (< 1.0 mg/d) dietary vitamin B$_6$ intake (279), 16%, 82%, and 84% of pregnant women had plasma PLP concentrations < 30 nmol/L in the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ trimesters, respectively (279). Another study in Japan reported 100% of pregnant women had plasma PLP concentrations < 30 nmol/L during pregnancy, with mean (± SD) plasma PLP concentrations of 5.1 (± 3.4) and 4.7 (± 6.0) nmol/L in the 2$^{nd}$ and 3$^{rd}$ trimesters, respectively (194). The prevalence of plasma PLP deficiency (<20 nmol) during pregnancy in a multi-ethnic population in Singapore was 16% (19). However, in folate-replete
Canada where prenatal supplementation is common and voluntary fortification is permissible, vitamin B₆ deficiency (< 20 nmol/L) during pregnancy was reported to be 0% and < 0.2% in the first and second trimesters, respectively (192). Data from the MoBa Study in Norway (n=2911) reported folic acid containing supplement users had significantly higher plasma PLP concentrations at 18 weeks gestation [median (IQR): 29.3 (21.9–42.0) nmol/L] compared to non-users [24.1 (18.6–30.4) nmol/L] (p<0.001) (280). A sole observational study investigating PLP concentrations in infants reported higher cord plasma PLP than maternal concentrations; In full term infants, the mean (± SD) cord plasma PLP concentration was 64 ± 39 nmol/L compared to 7 ± 4 nmol/L in maternal plasma (281). Overall, there is limited recent North American data on PLP concentrations in maternal and cord blood. Assessing PLP status in this population is of considerable interest given vitamin B₆ is voluntarily added to flour in North America (282) and prenatal supplements containing vitamin B₆ are widely used (189, 192).
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>USA</td>
<td>USA</td>
<td>Taiwan</td>
<td>Japan</td>
<td>Norway</td>
<td>Japan</td>
<td>Canada</td>
<td>Singapore</td>
</tr>
<tr>
<td><strong>Study design</strong></td>
<td>Experimental</td>
<td>Experimental</td>
<td>Observational</td>
<td>Experimental</td>
<td>Observational</td>
<td>Observational</td>
<td>Observational</td>
<td>Observational</td>
</tr>
<tr>
<td><strong>Sample size</strong></td>
<td>26</td>
<td>196 at &lt;22 wks, 46 at 30 wks, 22 at delivery</td>
<td>31</td>
<td>209</td>
<td>10 in 2nd tri, 18 in 3rd tri</td>
<td>2911</td>
<td>406</td>
<td>119 in 1st tri, 528 in 2nd tri</td>
</tr>
<tr>
<td><strong>B6 sup?</strong></td>
<td>2.5, 4, or 10 mg/d</td>
<td>Yes, 0, 2.6, 5, 7.5, 10, 15 or 20 mg of PN-HCl (no Bendectin)</td>
<td>Not addressed</td>
<td>0, 1, 2, or 3 mg/d</td>
<td>PN-HCl</td>
<td>No</td>
<td>Folic acid supp use</td>
<td>No</td>
</tr>
<tr>
<td><strong>PLP assay</strong></td>
<td>Enzymatic</td>
<td>Enzymatic</td>
<td>HPLC</td>
<td>HPLC</td>
<td>Enzymatic</td>
<td>HPLC</td>
<td>HPLC</td>
<td>HPLC</td>
</tr>
<tr>
<td><strong>Time of blood draw</strong></td>
<td>every six wks, childbirth</td>
<td>&lt;22 wks, 30 wks gestation, at term, cord blood</td>
<td>childbirth</td>
<td>childbirth</td>
<td>26-29 wks, 34-36 wks</td>
<td>18 wks</td>
<td>1st, 2nd, 3rd, tri, 1 mo after birth</td>
<td>≤13 wks, 14-26 wks</td>
</tr>
<tr>
<td><strong>Fasted samples?</strong></td>
<td>Not addressed</td>
<td>No</td>
<td>Not addressed</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Maternal PLP, mean ± SD</strong></td>
<td>15.4 ± 3.2 nmol/L at childbirth</td>
<td>&lt;22 wks: 37.1 ± 25.3 nmol/L (before supp), Delivery: 12.3 ± 9 nmol/L for 0-5 mg/d, 38 ± 25 nmol/L for 7.5-20 mg/d</td>
<td>7.3 ± 3.8 nmol/L</td>
<td>18 ± 6 nmol/L for 0mg/d, 20 ± 9 nmol/L for 1mg/d, 43±10 nmol/L for 2 mg/d, 58±8 nmol/L for 3mg/d</td>
<td>5.1 ±3.4 nmol/L in 2nd tri, 4.7 ±6.0 nmol/L in 3rd tri</td>
<td>Median (IQR) 29.3 (21.9–42.0) nmol/L in users, 24.1 (18.6–30.4) nmol/L in nonusers</td>
<td>56 ± 32 nmol/L, 22 ± 14 nmol/L, 19 ± 13 nmol/L, 42 ± 31 nmol/L in 1st, 2nd, 3rd tri and 1 mo after birth</td>
<td>Median (95CI) 94 (82, 112) nmol/L for 1st tri, 76 (70, 83) nmol/L in 2nd tri</td>
</tr>
<tr>
<td><strong>Cord PLP, mean ± SD</strong></td>
<td>~160, 220, and 320 nmol/L for 2.5, 4, and 10 mg/d</td>
<td>85.5 ± 45 nmol/L for 0-5 mg/d, 153 ± 54 nmol/L for 7.5-20 mg/d</td>
<td>64±39 nmol/L</td>
<td>20±6 nmol/L for 0mg/d, 40±8 nmol/L for 1mg/d, 78±2 nmol/L for 2 mg/d, 90±7 for 3mg/d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Vitamin B₆ is recognized as a prophylactic measure against nausea and vomiting in pregnancy (283), although its mechanism of action has not been elucidated. It appears that PLP is the active antiemetic form of vitamin B₆ (284). It has been used in prescription medications since the 1950’s with the introduction of Bendectin® in the US (285). In Canada, the drug is sold under the trade name Diclectin® (Diclegis® in the US) (Duchesnay Inc.) and the formulation consists of 10 mg pyridoxine hydrochloride combined with doxylamine, an antihistamine. Individual studies have shown Diclectin® is effective in subsiding the effects of “morning sickness” (286-288). However, a recent meta-analysis did not find a significant protective effect of Diclectin® against pregnancy-associated nausea and vomiting (289). Following the standard dosage, users may consume up to 4 tablets of Diclectin® a day, totaling up to 40 mg/d pyridoxine hydrochloride (290). This is a significant source of vitamin B₆ considering most prenatal vitamins available on the Canadian market only contain 1.9 mg/d of vitamin B₆, the RDA of vitamin B₆ during pregnancy (1).

2.3.7.3 Maternal vitamin B₆ status and Health Outcomes in the Offspring

Several studies investigating the association between maternal vitamin B₆ status and health outcomes in the offspring have reported equivocal and inconsistent results. Two early experimental studies investigated the effects of maternal vitamin B₆ status on Apgar scores in the neonate. Schuster et al. reported abnormally high RBC alanine aminotransferase (ALT) activity (an indicator of vitamin B₆ deficiency) was associated with lower Apgar scores at 1 minute (36). However, this population consisted mainly of pregnant women from a low socio-economic background with an average vitamin B₆ intake of (mean ± SD) 1.37 ± 1.02 mg (36). In a dose responsive clinical study, the same group of investigators also reported in pregnant women with
initial PLP concentrations of 37.1 ± 25.3 nmol/L at approximately 15 weeks gestation, maternal vitamin B$_6$ supplementation of at least 7.5 mg/d pyridoxine hydrochloride was necessary to maintain PLP concentrations at early pregnancy levels during pregnancy and this supplemental level was associated with higher Apgar scores at 1 minute compared with the lower levels (15).

A large case-control study involving 2,013 cases and 4,086 controls reported that the use of pyridoxine during pregnancy was associated with a protective effect against cardiovascular malformations (adjusted prevalence OR 0.8; 95% CI: 0.7, 0.9) (291).

An Australian case-control study investigated the association between maternal dietary one-carbon nutrients, including vitamin B$_6$, in the last 6 months of pregnancy and the risk of childhood acute lymphoblastic leukemia (ALL) (292). While higher intakes of folate and vitamin B$_{12}$ had a protective effect, there was an increased risk of childhood ALL associated with higher maternal vitamin B$_6$ intakes. However, other studies have reported a protective effect of maternal vitamin B$_6$ status on other cancers (292). Two case control studies have reported that maternal (196) or childhood (293) vitamin B$_6$ intake was not associated with childhood brain tumors.

Regarding vitamin B$_6$ exposure through the use of Diclectin® during pregnancy, a Canadian prospective study reported children born to mothers who used this antiemetic drug to combat nausea and vomiting during pregnancy scored significantly higher on performance IQ and pictorial memory tests (at 3-7 years of age) and experienced fewer sleep problems than children born to mothers who did not use Diclectin® (294). However, there are several limitations associated with this study. Maternal hormonal factors can influence neurodevelopment in the offspring (295) but this study did not take into consideration potential hormonal differences between women who experienced nausea and vomiting during pregnancy and those who did not,
which may have confounded the observation. Furthermore, it is difficult to conclude which ingredient, pyridoxine hydrochloride or doxylamine or both, was responsible for the observed beneficial effect. Most importantly, no biologically plausible mechanisms of Diclectin® exist to account for the improved neurodevelopment in the offspring.

Overall, more research is needed to understand the association between maternal vitamin B₆ and health outcomes in the offspring. Exposure to high levels of vitamin B₆ during pregnancy is possible with the use of vitamin B₆ containing anti-emetics and commonly used supplements, yet inadequate blood levels are still being detected (279, 280). This fact underscores the need to characterize vitamin B₆ status during pregnancy in Canada, as little data currently exist.

2.4 Choline and Betaine

2.4.1 Definition, chemical structure, and dietary sources

Choline is naturally found in the food supply as free choline, or bound as esters such as phosphocholine, glycerophosphocholine, sphingomyelin or phosphatidylcholine (PC). Choline is an essential nutrient which functions as a precursor for the neurotransmitter acetylcholine and along with its derivatives, also serves as components of structural lipoproteins (such as phospholipids), blood and membrane lipids (1). Although it is considered an essential nutrient, choline can also be synthesized de novo by the endogenous pathway via methylation of phosphatidylethanolamine (PE) (Figure 2.8). This reaction is catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT) with SAM serving as the methyl donor.
Figure 2.8. Choline metabolism. BHMT, betaine homocysteine methyltransferase; CDP, cytidine diphosphate; CHDH, choline dehydrogenase; DMG, dimethylglycine; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; PEMT, phosphatidylethanolamine N-methyltransferase; THF, tetrahydrofolate; TMAO, trimethylamine N-oxide. Figure reprinted with permission from Zeisel, 2007 (296).

Although PEMT-catalyzed *de novo* biosynthesis of PC provides a significant source of choline, this does not provide an adequate amount to meet the nutrient requirements when concentrations of other one-carbon metabolism nutrients, namely methionine, folate, vitamin B₆ and B₁₂, are not available in sufficient amounts to sustain normal growth and function (1). This interrelationship underscores the importance of studying one-carbon nutrients together and adds the complexity to this field of research as it is often very difficult to tease apart effects of individual one-carbon nutrients.
Choline is found in foods as free choline and as phosphocholine, glycerophosphocholine, sphingomyelin, and PC, its esterified forms. Natural dietary sources of choline occurs naturally in egg yolks, beef, chicken, liver, and soybeans (1, 297, 298). A PC-rich compound used as a food emulsifying agent is called lecithin and is synonymous with PC (1).

![Chemical structures of choline and betaine](image)

**Figure 2.9. Chemical structures of choline and betaine.** From Ueland PM 2011 with permission (298).

Most choline is irreversibly oxidized to betaine in the liver and kidney in a two-step process by choline dehydrogenase and betaine aldehyde dehydrogenase. Betaine is a zwitterionic quaternary ammonium compound (Figure 2.9). It is a methyl derivative of the amino acid glycine and can be characterized as a methylamine because of its three chemically reactive methyl groups (299). Betaine is abundantly found in animal foods, especially seafood and plant foods such as wheat bran and spinach (300).

### 2.4.2 Absorption and Metabolism

The form of choline determines the mechanism of absorption and therefore choline
bioavailability (301). Dietary choline is absorbed in the small intestine by transporter proteins. Absorption of the lipid-soluble forms (PC and sphingomyelin) occur via the thoracic duct and bypass the liver, while the water-soluble forms (free choline, phosphocholine and glycerophosphocholine) enter portal circulation and are mostly absorbed by the liver (297). Pancreatic enzymes also hydrolyze dietary PC, phosphocholine and glycerophosphocholine to release free choline (1). Before dietary choline is absorbed in the small intestine, a portion is oxidized to form betaine by bacteria (302). Subsequently, free choline is absorbed in the lumen of the small intestine by transporter proteins in the enterocyte (1).

2.4.3 Biochemical Functions

Choline is important for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling, and lipid and cholesterol transport and metabolism (1, 298). Furthermore, choline is associated with the neurodevelopment and cell proliferation of the hippocampus, differentiation, and apoptosis in animals and is also believed to affect brain development and cognitive functions in humans (303).

Betaine participates as a substrate in the BHMT reaction (210). In this reaction occurring in the human liver and kidney (66), betaine serves as a methyl donor, by transferring a methyl group to homocysteine for the conversion to methionine, which is the focal point where betaine and choline are linked to one-carbon metabolism. On an intracellular level, betaine functions as an osmolyte that regulates cell volume and tissue integrity, thereby protecting cells and proteins from environmental stresses (299).
2.4.4 Choline and Betaine Intake Requirements

The Adequate Intake (AI) is used for choline and betaine since there is insufficient scientific evidence to establish an EAR on which the RDA would be based. The AI of choline for men and women 19 years and older is 550 mg/d and 425 mg/d, respectively (1). This value for men was established based on one study, which found this intake (approximately 500 mg/d or 7 mg/kg body weight/day) to prevent increases in hepatic alanine aminotransferase (indicative of liver inflammation) in healthy men (304). The AI for women has been extrapolated from the AI value established for men since no studies involving females currently exist. However, from animal studies, it is believed that choline requirements in females are less than males due to the increased capacity to synthesize choline de novo in females by estrogen-mediated mechanisms (1), although this enhancement may subside after menopause (1). The AI for pregnancy is slightly higher than the non-reproductive state. Although estrogen levels are elevated during pregnancy, which increase the activity of hepatic PEMT and hence, endogenous choline production (305), there is still a greater choline demand due to fetal and placental accumulation of choline. Large amounts of choline are delivered to the fetus via the placenta which may deplete maternal stores (1). Therefore, through estimated calculations of fetal and placenta weight and choline content and assuming no synthesis of choline from the fetus or placenta, an additional 11 mg/d was deemed to be needed (1). After rounding up, the AI is 450 mg/d for 14-50 year old pregnant females.

The UL for male and female adults is 3500 mg/d (1). The UL is based on two adverse outcomes associated with increased choline intake in humans: hypotension and fishy body odor (1). Excess choline intake may contribute to hypotension by increasing vagal tone to the heart or dilating
arterioles (1). Also, choline intakes above 3500mg/d can lead to excessive amounts of trimethylamine excretion, a choline metabolite, causing fishy body odor (1).

2.4.5 Biomarkers of Choline and Betaine

The main consequence of inadequate choline intake is fatty liver, which can lead to liver dysfunction (1). Therefore, choline deficiency is often diagnosed by measuring liver enzymes, including ALT, as a marker of liver inflammation and injury. However, ALT concentrations do not reflect choline status. Nonetheless, one study in Mexican American men, half with the MTHFR677 TT genotype, reported a choline intake of 550 mg/d (300 mg/d from diet and 250 mg/d from supplements) for nine weeks was adequate enough to prevent elevations in serum ALT concentrations (306).

Fasting plasma choline concentrations range from 7 to 20 µmol/L, with 10 µmol/L as the most common concentration for individuals (1). The use of plasma choline concentration as a diagnostic tool has its limitations since choline is physiologically regulated (1). For example, concentrations do not appear to decrease below approximately 50%, even when fasting for more than a week (1). Plasma betaine concentrations are not routinely measured, although its measurement in conjunction with plasma choline, dimethylglycine (DMG) and trimethylamine N-oxide (TMAO) offer a more comprehensive assessment of overall choline status and metabolism.
2.4.6 Choline and Betaine and Health

Inadequate choline intakes leads to fatty liver, which results from a lack of PC, and consequent decreased export of excess triacylglycerols from the liver (307). Both choline and betaine are considered lipotropes based on their ability to prevent fatty liver (308). Other signs of choline deficiency are liver and muscle damage (297, 298, 307). Sex and menopausal status have been shown to influence dietary requirements and blood levels of choline (1). Premenopausal women are less susceptible to choline deficiency and consequent fatty liver and liver damage compared with men and postmenopausal women owing to the estrogen-mediated upregulation of the de novo synthesis of choline (309). Furthermore, because choline is involved in the remethylation pathway of homocysteine, choline deficiency can result in increased levels of plasma homocysteine (298). Although the homocysteine-lowering effect of choline and betaine is well documented, there is conflicting evidence concerning the potential of these two nutrients to modulate the risk of coronary heart disease and stroke via the homocysteine-lowering effect (1).

2.4.7 Choline and Betaine in Pregnancy

2.4.7.1 Role of Choline and Betaine in Pregnancy Outcomes

Choline has an important function in the very early stages of pregnancy as it contributes to the closure of the neural tube and growth of maternal organs such as the kidneys, uterus, and the placenta (210, 310). The developing fetus has a high demand for choline, which is required for the cell membrane synthesis, neurotransmission and brain development. In particular, during the late stages of pregnancy when fetal organ growth is rapid, large amounts of choline are required for membrane biosynthesis and the neurogenesis of the fetal hippocampus (210, 310). Since the
placenta and fetal liver do not express the PEMT enzyme necessary for endogenous choline production, maternal choline stores are the sole source of fetal choline. The placenta accumulates choline and delivers it to the fetus via diffusion against its concentration gradients (311). Inadequate choline has been shown to be a risk factor for NTDs, independent of folate (21, 26). Suboptimal choline concentrations may disrupt embryonic DNA methylation, cell membrane synthesis or impair regulation of apoptosis, all contributing to the increased risk for NTDs (26). Increased maternal dietary choline intake during pregnancy is also associated with reduced risk of orofacial clefts (23), hypospadias (27) and congenital diaphragmatic hernia (25). Although only a few studies have investigated this, increased choline intake during pregnancy has also been shown to lower maternal homocysteine concentrations, thus lessening the risk of adverse pregnancy outcomes associated with elevated maternal homocysteine concentrations, including preeclampsia, preterm labor, and low birth weight (312).

2.4.7.2 Choline and Betaine Exposure in Pregnancy

There is some concern that a large proportion of the population has suboptimal choline intakes. This is of particular concern for pregnant women who may not be meeting the choline demands of the developing fetus (297, 310). However, very little data exists on the choline status of non-pregnant and pregnant women. A cross-sectional analysis in over 1400 women from the Nurse’s Health Study reported the median intake of total choline and betaine was 323 and 189 mg/d, respectively (313). This is well below the AI of 425 mg/d for women (1). A conference abstract on choline intakes during pregnancy using 2003-2004 NHANES data reported 90% of women during pregnancy have choline intakes below the AI (314). Non-fasting plasma choline concentration was (mean ± SD) 8.4 ± 0.4 µmol/L in a small sample (n=16) of pregnant Jamaican
women at 10-15 weeks gestation (315). Data from a Canadian cohort of pregnant women reported median (IQR) concentrations of plasma choline at 16 and 36 weeks gestation to be 6.7 (5.8 - 8.0) and 9.4 (8.1 - 11.3) μmol/L, respectively (28). Median (IQR) betaine concentrations in the same population at 16 weeks gestation was 12.4 (10.4–15.1) μmol/L (28). More research is needed on the choline status of pregnant women considering adequate choline has been shown to reduce the risk of NTDs and is important in fetal neurocognitive development.

2.4.7.3 Maternal status of Choline and Betaine and Health Outcomes in the Offspring

Maternal dietary choline intakes or choline concentrations appear to influence the offspring’s cognitive development. Two studies from the prospective Project Viva study in the US have investigated the role of maternal choline intakes on cognitive function in the offspring with mixed results (29, 316). Among the one-carbon nutrients considered (folate, vitamin B₁₂, choline, betaine and methionine), only higher maternal folate intake during the first and second trimesters was found to have a beneficial effect on receptive language assessments in the children at three years of age (316). In contrast, Boeke et al. reported gestational choline intake, but not folate, vitamin B₁₂ or betaine intake, during the first and second trimesters was associated with modestly better child visual memory at seven years of age (29). These findings suggest the effects of maternal choline on cognitive function in the offspring may not be apparent until later childhood development.
A sole randomized, double-blind, placebo-controlled trial found that supplementing pregnant women with 750 mg/d PC from week 18 of gestation to 90 days postpartum had no beneficial effect on the cognitive abilities of their offspring at 10 and 12 months of age (317). A prospective study conducted in Canada reported maternal plasma concentrations of choline and betaine at 16 weeks was positively associated with cognitive development in the offspring at 1.5 years of age (28). However, maternal serum choline concentrations between 16-38 weeks gestation or in cord blood did not have an effect on childhood cognition at five years of age (318).

Considering choline is essential in many biological functions related to membrane integrity and cognitive development, it is not surprising that maternal choline intake and blood levels may have significant effects on birth outcomes, neurocognitive development and health outcomes of the offspring, although these effects have not been unequivocally demonstrated to date. Further research is well justified to clarify the role of maternal choline and betaine status on cognitive development and health outcomes in the offspring.

2.5 Genetic variants in one-carbon nutrient metabolism

A list of single nucleotide polymorphisms and other genetic variants assessed in the PREFORM Study are listed in Table 2.10.

Table 2.10. List of specific single nucleotide polymorphisms and other genetic variants assessed in the PREFORM Study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHMT</td>
<td>Betaine homocysteine methyltransferase</td>
<td>Encodes the enzyme BHMT which catalyzes the transfer of a methyl group from betaine to homocysteine forming methionine and dimethylglycine</td>
</tr>
<tr>
<td>Gene</td>
<td>Name</td>
<td>Function</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CβS</td>
<td>Cystathionine β-synthase</td>
<td>Encodes the enzyme CβS which catalyzes homocysteine to cystathionine, the first step in the transsulfuration pathway</td>
</tr>
<tr>
<td>CD320</td>
<td>Cluster of differentiation 320 (aka TChBII-R)</td>
<td>Encodes for the transcobalamin receptor that is expressed at the cell surface. It mediates the cellular uptake of transcobalamin bound vitamin B_{12}.</td>
</tr>
<tr>
<td>CHDH</td>
<td>Choline dehydrogenase</td>
<td>Encodes the enzyme CHDH which catalyzes the irreversible oxidation of free choline to betaine</td>
</tr>
<tr>
<td>CUBN</td>
<td>Cubilin</td>
<td>Encodes the protein cubilin, a nontransmembrane, multiligand receptor, which forms part of the cubam receptor in the ileum and is required for vitamin B_{12} absorption.</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
<td>Encodes for the enzyme DHFR which catalyzes the conversion of inactive folic acid to active DHF and subsequent THF in a two step process.</td>
</tr>
<tr>
<td>DPEP1</td>
<td>Dipeptidase 1</td>
<td>Encodes for a kidney membrane enzyme involved in the metabolism of glutathione and other similar proteins by dipeptide hydrolysis.</td>
</tr>
<tr>
<td>FUT2</td>
<td>α(1,2)-fucosyltransferase</td>
<td>Encodes the α(1,2)-fucosyltransferase enzyme that mediates the formation of H type 1 and 2 antigens which regulate the adhesion of the gastric pathogen, Helicobacter pylori, to the gastric and duodenal mucosa. Overgrowth of Helicobacter pylori is associated with vitamin B_{12} deficiency.</td>
</tr>
<tr>
<td>GGH</td>
<td>Gamma glutamyl hydrolase</td>
<td>Encodes for the enzyme, which catalyzes the hydrolysis of folylpoly-gamma-glutamates and antifolylpoly-gamma-glutamates by the removal of gamma-linked polyglutamates and glutamate.</td>
</tr>
<tr>
<td>MMAA</td>
<td>Methylmalonic aciduria type A protein</td>
<td>Encodes the MMAA protein, which enables the translocation of vitamin B12 into the mitochondrion where it is used in the final steps of adenosylcobalamin synthesis.</td>
</tr>
<tr>
<td>MTHFD1</td>
<td>Methylene tetrahydrofolate dehydrogenase</td>
<td>Encodes the enzyme MTHFD1 which converts 10-formylTHF to 5,10-methyleneTHF.</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
<td>Encodes the enzyme MTHFR which converts 5,10-methyleneTHF to 5-methylTHF.</td>
</tr>
<tr>
<td>MTR</td>
<td>5-Methyltetrahydrofolate-homocysteine methyltransferase</td>
<td>Encodes the enzyme methionine synthase which catalyzes the transfer of a methyl group from 5-methylTHF to homocysteine forming methionine and regenerating THF.</td>
</tr>
<tr>
<td>MTRR</td>
<td>5-Methyltetrahydrofolate-homocysteine methyltransferase reductase</td>
<td>Encodes the enzyme methionine synthase reductase which is required for the regeneration of methionine synthase.</td>
</tr>
<tr>
<td>MUT</td>
<td>Methylmalonyl-CoA mutase</td>
<td>Encodes the enzyme MUT which converts L-methylmalonyl-CoA to succinyl-CoA.</td>
</tr>
<tr>
<td>NOX4</td>
<td>NADPH Oxidase 1</td>
<td>Encodes for a member of the NOX-family of enzymes that functions as the catalytic subunit the NADPH oxidase complex.</td>
</tr>
<tr>
<td>Gene</td>
<td>Name</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
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<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PEMT</td>
<td>Phosphatidylethanolamine N-methyltransferase</td>
<td>Encodes the enzyme PEMT which converts PE to PC</td>
</tr>
<tr>
<td>PON1</td>
<td>Paraoxonase 1</td>
<td>Encodes for a protein involved in preventing the toxic effects of homocysteine due to its homocysteine hydrolase activity.</td>
</tr>
<tr>
<td>RFC1</td>
<td>Replication factor C (activator 1)</td>
<td>Encodes for a DNA polymerase accessory protein involved in DNA replication and repair</td>
</tr>
<tr>
<td>TCN1</td>
<td>Haptocorrin</td>
<td>Encodes the transport protein haptocorrin which carries 70-80% of total circulating vitamin B12, but does not deliver it to tissues</td>
</tr>
<tr>
<td>TCN2</td>
<td>Transcobalamin</td>
<td>Encodes the transport protein transcobalamin which carries 20-30% of total circulating vitamin B12 and delivers it to tissues</td>
</tr>
<tr>
<td>TRDMT1</td>
<td>tRNA aspartic acid methyltransferase 1</td>
<td>Encodes for the protein responsible for the methylation of aspartic acid transfer RNA, specifically at the cytosine-38 residue in the anticodon loop.</td>
</tr>
</tbody>
</table>

2.5.1 Genetic variants in folate metabolism

Genetic variants in two major enzymes involved in folate metabolism, MTHFR and DHFR, can significantly alter folate concentrations and disease risk. As previously mentioned, intracellular folate homeostasis depends on the critical enzyme MTHFR that catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF. A single nucleotide polymorphism (SNP) (C→T substitution at nucleotide position 677) (rs1801133) in the MTHFR gene, which results in an alanine to valine substitution, is associated with reduced MTHFR activity and increased thermolability of MTHFR (319). This mutation can lead to decreased 5-methylTHF and an accumulation of 5,10-methyleneTHF in RBCs. The MTHFR C677T is a common mutation, with an allele frequency of about 35% in the general North American population (1). However, the frequency of the MTHFR C677T polymorphism varies among racial and ethnic groups; the TT genotype is common in Hispanics (320) while African-Americans exhibit very low prevalence of the TT genotype compared to Caucasians and Asians (63, 321). Because the mutation is
associated with reduced MTHFR activity, some, but not all, studies have shown that the \textit{MTHFR} C677T polymorphism is associated with an increased risk of cardiovascular disease, NTDs, adverse pregnancy outcomes, Down’s syndrome, neuropsychiatric disorders and cognitive impairment, and cancer (322). The risk modification rendered by the \textit{MTHFR} C677T polymorphism seems dependent on the folate status. (322).

Several studies have investigated how the \textit{MTHFR} C677T polymorphism affects both folate and homocysteine concentrations. The Norwegian Colorectal Cancer Prevention (NORCCAP) study involving over 10,000 subjects investigated the effects of 13 genetic variants involved in one-carbon metabolism on biochemical parameters. The strongest associations were observed for the \textit{MTHFR} \textit{C677T} SNP. Plasma homocysteine increased (\textit{p}<0.001), whereas plasma folate decreased (\textit{p}<0.001) in a dose-responsive manner as the number of the T allele increased (323). Lower serum folate concentrations have been observed in individuals with TT genotype in a young female (324) and an adult male and female (325) Japanese population. A few studies indicate that individuals homozygous for the mutation (TT genotype) have mildly elevated plasma total homocysteine concentrations compared with individuals with either the heterozygous (CT) or homozygous wild-type (CC) genotype (326). However, it seems that elevated plasma levels of homocysteine are more consistently observed in individuals with the TT genotype under conditions of inadequate folate status (327). When folate status is adequate, plasma homocysteine levels are generally not affected by the \textit{MTHFR} C677T genotype (328, 329). A recent meta-analysis of intervention studies confirmed those with the TT genotype had higher homocysteine and lower serum folate concentrations compared to those with the CT or CC genotypes (330).
Another common SNP in the MTHFR gene (A→C substitution at nucleotide position 1298) (rs1801131) results in a glutamine to alanine substitution, and is also associated with reduced MTHFR activity, although its effect is more subtle than the C677T SNP (322). Unlike the C677T, the A1298C SNP does not result in a thermolabile enzyme (322). The A1298C variant is not as widely studied at C677T. However, the majority of studies have not found the A1298C variant to be independently associated with NTDs or other birth defects such as congenital heart disease and cleft lip and palate (322). In a recent study in China, the A1289C variant was found to be protective against NTDs, which was supported by a meta-analysis conducted by the same group (331). In the NORCCAP Study, the A1298C SNP affected plasma homocysteine and folate; homocysteine increased (p<0.001), while folate decreased (p<0.001) in a dose responsive fashion with the number of the C allele (323).

Another important enzyme involved in folate metabolism is DHFR, which converts folic acid to THF. The most widely studied DHFR polymorphism is a 19 base pair deletion occurring within intron 1 (rs70991108); however, its functional outcomes are still unclear. In the Framingham cohort study (n=1215), individuals with the del/del genotype who consumed < 250 µg/d folic acid had lower RBC folate concentrations compared to the ins/ins and ins/del genotypes (332). Furthermore, individuals with the del/del genotype consuming greater than 500 µg/d folic acid had significantly higher UMFA concentrations compared to the homozygous wild-type and heterozygous genotypes, indicating a lower capacity for DHFR to convert folic acid to DHF (332). On the contrary, a smaller study found higher RBC folate concentrations with the del/del genotype, but only in women, not men (333). Other studies have found lower homocysteine in men and women (334) or no association in men and women (335) and women only (336) with
the del/del genotype. The del/del polymorphism has also been associated with decreased risk of NTDs (333, 337), although the results are inconsistent (338, 339). Finally, a decreased risk of ALL was found with the del/del genotype (340) but no association was found with sporadic colon cancer (341).

2.5.2 Genetic variants in vitamin B\textsubscript{12} metabolism

Although there were no reported effects of any of the 13 genes on vitamin B\textsubscript{12} concentration in the NORCCAP Study, MMA concentrations were positively associated with two TCN2 polymorphisms (776C>G and 67A>G) (323). This relationship is biologically plausible since the TCN2 polymorphisms lead to reduced vitamin B\textsubscript{12} transportation via reduced transcobalamin activity, causing less intracellular vitamin B\textsubscript{12} and a consequent rise in MMA (323). Two studies demonstrated individuals homozygous for the FUT2 A>G (rs602662) polymorphism have higher vitamin B\textsubscript{12} concentrations compared to wildtype or heterozygotes (342, 343). Conversely, mutations in the TCN1 gene, which encodes for haptocorrin, are associated with lower vitamin B\textsubscript{12} concentrations (344).

A comprehensive study of SNP data was recently published from a Canadian cross-sectional study examining associations between 116 common SNPs in genes related to folate and vitamin B\textsubscript{12} metabolism and biomarkers of nutrient or disease-related status in a folic acid fortified population (345). This study identified two SNPs in FUT2 (rs492602 and rs602662) were linked to less risk for deficient (<148 pmol/L) or inadequate (<220 pmol/L) vitamin B\textsubscript{12} status and were associated with higher serum vitamin B\textsubscript{12} concentrations. SNPS in the \textit{CβS} (rs21244590), \textit{CD320} (rs2336573), \textit{CUBN} (rs112543630), \textit{DNMT2} (rs22958090) and \textit{PON1} (rs3917577) genes
were less likely to have vitamin B\textsubscript{12} \textless 220 pmol/L, and the CD320 and DNMT2 SNPs were associated with higher serum vitamin B\textsubscript{12}. On the other hand, the TCNI (rs526934) and TCN2 (rs757874) SNPs were associated with a higher risk for vitamin B\textsubscript{12} status \textless 220 pmol/L (345). Collectively, these results suggest genetic variants in vitamin B\textsubscript{12} metabolism do play a functional role in modulating concentrations of vitamin B\textsubscript{12} and its biomarkers.

2.5.3 Genetic variants in vitamin B\textsubscript{6} metabolism

Two large-scale studies have reported alterations in plasma PLP concentrations and other metabolites (i.e. homocysteine and cystathionine) related to vitamin B\textsubscript{6} with various genetic polymorphisms. Of the 13 SNPs analyzed in the NORCCAP Study, the T variant in the MTHFR C\textsubscript{677}T SNP was associated with a significant increase in total homocysteine and a decrease in plasma PLP (323). The C\beta\textsubscript{84} 844\text{ins}68 (68 base pair insertion) and C\beta\textsubscript{84} 699C>T polymorphisms were both negatively associated with homocysteine. Although the decrease in homocysteine was suggestive of increased C\beta\textsubscript{84} enzyme activity with the C\beta\textsubscript{84} 844\text{ins}68 polymorphism, the concomitant increase in betaine suggests homocysteine is being remethylated rather than rapidly cleared by C\beta\textsubscript{84} (323). The C\beta\textsubscript{84} 699C>T SNP was also positively associated with cystathionine, the intermediary metabolite in the vitamin B\textsubscript{6} dependent transsulfuration pathway. Another genome-wide association study, which combined data from three large populations and confirmed the findings in an independent population, investigated genetic factors that affect circulating PLP and other one-carbon metabolites (342). Relating to vitamin B\textsubscript{6}, the C variant of the ALPL C>T (rs4654748) SNP was associated with lower serum PLP concentrations. ALPL encodes for the alkaline phosphatase enzyme, which removes phosphate groups from various molecules, including vitamin B\textsubscript{6} (342). Mutations in this gene are associated with a genetic
disease characterized by low or complete lack of alkaline phosphatase activity and results in the accumulation of vitamin B₆ (PLP) (342). Therefore, the lower serum PLP associated with the variant C is most likely a result of more efficient vitamin B₆ clearance (342).

2.5.4 Genetic variants in choline metabolism

Polymorphisms in one-carbon metabolism related genes may alter choline requirements and circulating betaine concentrations. In the population-based NORCCAP Study investigating genetic variants in 13 genes on biochemical parameters, the MTHFR C677T (rs1801133) SNP was associated with decreased betaine and DMG concentrations and there was a dose-response relationship as the number of the T allele increased (323). Likewise, the MTHFR A1298C (rs1801131) SNP was associated with decreased betaine concentrations and the effect was larger in those homozygous for the variant (CC) compared to heterozygotes. The G716A (rs3733890) SNP in BHMT led to a significant decrease in DMG and the effect size increased as the number of the A allele increased. Finally, the T variant in the CβS 844ins68 and C699T polymorphisms was positively associated with betaine concentrations (323). Interestingly, none of the SNPs investigated in this study demonstrated a significant association with plasma choline concentrations.

In a controlled feeding study of young Mexican American women with a high prevalence of the MTHFR 677TT (rs1801133) genotype, women with the TT genotype had higher PC concentrations compared with those with the CT genotype but had similar concentrations to those with the CC genotype (346). Mutations in other one-carbon nutrient related genes, including PEMT G774C (rs12325817) (347, 348), CHDH T233G (rs12676) (347) and MTHFD1
G1958A (rs2236225) (349), were positively linked to an individual’s susceptibility for choline deficiency. In this study, clinical choline deficiency was defined as more than five times increase in serum creatine kinase activity or a >28% increase of liver fat (diagnosed by magnetic resonance imaging scan) after consuming a low-choline diet that resolved when choline was returned to the diet (347). The A119C mutation in the CHDH gene (rs9001) (347) was found to have a protective effect against developing choline deficiency under the same feeding conditions.

A few studies have examined the effects of SNPs in one-carbon or choline metabolism on concentrations of choline and its metabolites during pregnancy. Fischer et al. genotyped pregnant women for 370 common SNPs in 10 genes and randomize them to either a daily 750 mg choline supplement or placebo from week 18 gestation to 45 days postpartum. This study found five SNPs in the MTHFR gene (rs1537516, rs17367629, rs3753582, rs3753588, rs6687229) interacted with dietary choline intake to alter breast milk choline concentrations, while a SNP in the PEMT (rs711352) gene was associated with increased betaine concentrations in breast milk (350). However, none of the 370 SNPs affected plasma choline concentrations or interacted with dietary choline intake to alter plasma choline concentrations (350).

2.6 DNA Methylation and Hydroxymethylation

2.6.1 DNA Methylation: Definition, functions, and biological significance

Epigenetics refers to heritable changes in phenotype without changes in genotype (351). Epigenetic mechanisms include DNA methylation, covalent modifications of histones, chromatin
remodeling, and RNA interference, all of which alter gene expression and function without changing the nucleotide sequence (352). In contrast to genetic changes in human diseases, epigenetic changes are gradual in onset and progressive, their effects are dose-dependent, and are potentially reversible by dietary and pharmacologic manipulations (62, 353). One of the main epigenetic DNA modifications in mammals is the methylation of cytosine located within the cytosine-guanine dinucleotide (CpG) sequences, which is heritable, and tissue- and species-specific (354, 355). DNA methylation is an important epigenetic determinant in gene expression, in the maintenance of DNA integrity and stability, and in chromatin modifications (356). Up to 80% of all CpG sites in human DNA are normally methylated, however, this methylation occurs primarily in the bulk of the genome where CpG density is low (357). By contrast, most CpG rich areas clustered in small stretches of DNA termed “CpG islands”, which span the 5’ end of approximately half of the human genes including the promoter, untranslated region, and exon 1, are unmethylated in normal cells, thereby allowing transcription to occur. When methylated, CpG islands can cause stable heritable transcriptional silencing or in certain cases, activation (358). Silencing is mediated by transcriptional repressors, methyl-CpG binding protein-2 (MeCP2) and methyl-CpG binding proteins (MBD) 1, 2, and 4 (359), which bind methylated CpG islands and recruit a complex containing a transcriptional co-repressor and a histone deacetylase (HDAC) (360, 361). Deacetylation of histones suppresses transcription by allowing tighter nucleosomal packaging and thus rendering an inactive chromatin conformation (362, 363).

DNA methylation is a dynamic process between active methylation, mediated by CpG methyltransferases (DNMT1, 3a, 3b) (364) using SAM (365), and removal of methyl groups
from 5-methylcytosine (5mC) residues by several mechanisms, including active demethylation by ten eleven translocation (TET) proteins (366). Regulation of DNA methylation is largely dependent on SAM-dependent methyltransferases and SAH hydrolase (SAHH) (367). SAM is converted to SAH by SAM-dependent methyltransferases and SAH is hydrolyzed to homocysteine by SAHH. Although SAHH strongly favors SAH synthesis, the reaction equilibrium is towards homocysteine synthesis due to efficient removal of homocysteine by multiple pathways (CβS and MS) (368). Effective clearance of SAH and homocysteine are very important given accumulation of SAH acts as an inhibitor of DNMTs (367) and has been shown to upregulate CβS and downregulate MTHFR, MS and BHMT (368). Furthermore, elevated SAH concentrations have been associated with DNA hypomethylation in animal (369) and human (370, 371) studies.

DNA methylation is critically involved in regulating many developmental and cellular processes including embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, and chromosome stability (372). Aberrant patterns and dysregulation of DNA methylation are mechanistically related to the development of several human diseases including asthma (373), cancer (5, 355, 356, 374), cardiovascular disease (375), and metabolic disorders (376, 377). Cancer, in particular, has been shown to have distinct epigenetic characteristics that appear early on in cancer initiation and progression. Cancer is characterized by global DNA hypomethylation as well as gene-specific CpG promoter DNA hypermethylation of generally unmethylated CpG islands in the promoter region of tumor suppressor genes, leading to transcriptional gene silencing (62) (Figure 2.10). Global DNA hypomethylation contributes to the development of cancer through a number of mechanisms including chromosomal instability, increased mutations, reactivation of intragenomic parasitic
sequences, loss of heterozygosity and promotion of rearrangements, aneuploidy, loss of imprinting, and upregulation of proto-oncogenes (374). DNA methylation at promoter CpG islands silences transcription, thereby inactivating the function of a wide array of genes that have classic tumor suppressor function or play critical roles in cancer development and progression (357). Furthermore, cancer has also been shown to be characterized by overexpression of HDACs which is linked to gene silencing due to the inaccessibility of transcriptional factors to bind to DNA (378).

**Figure 2.10. Global and gene-specific methylation patterns in normal and cancer cells.** A) In normal cells, methylation generally occurs globally; CpG islands in promoter regions of genes are unmethylated, allowing for transcription. B) In cancer, global hypomethylation and CpG promoter region hypermethylation lead to blockage of transcription and silencing of gene expression. Modified from Masih et al. 2014 (379).
2.6.2 DNA Hydroxymethylation: Definition, functions, and biological significance

DNA methylation had long been thought as stable, irreversible epigenetic markers. However, the discovery of TET proteins and their effects on 5-methylcytosine (5mC) suggest DNA methylation may be more susceptible to environmental factors than previously thought (380). TET proteins are responsible for oxidation of 5mC to 5hmC, a marker for active DNA demethylation. 5hmC can further be converted to 5-formylcytosine and 5-carboxylcytosine, and ultimately back to an unmodified cytosine base pair by TET proteins and the thymine DNA glycosylase (TDG)-mediated base excision repair (358) (Figure 2.11).

Figure 2.11. Simplified scheme of the conversion of active DNA demethylation. 5-methylcytosine is oxidized to 5-hydroxymethylcytosine by TET 1, 2, and 3. 5-hydroxymethylcytosine is converted back to an unmodified cytosine through a number of conversions mediated by TET and thymine DNA glycosylase (TDG)-mediated base excision repair (BER).
Besides functioning as transitional markers of active DNA demethylation, emerging evidence suggests that 5hmC may potentially influence transcription by attracting or repelling specific DNA-binding proteins (358). The biological functions of 5hmC are not fully understood, yet recent advances indicate 5hmC may play a very important role in diverse biological processes such as embryonic and germ cell development and cancer (358). The role of 5mC and 5hmC in human embryonic development will be discussed below.

### 2.6.3 Epigenetic programming

As mentioned previously, DNA methylation is a dynamic process between active methylation and removal of methyl groups from 5mC residues. During human embryogenesis, active and passive demethylation of the paternal and maternal methylation patterns, respectively, occurs, which erases significant parts of the parental DNA methylation blueprint (381). This is followed by de novo methylation, which establishes a new DNA methylation pattern soon after implantation, with methylation limited to non-CpG island areas, except for the rare genes silenced in normal cells (Figure 2.12) (364, 381). Simultaneously, because the paternal methylome is actively erased, there is an increase in 5hmC in the paternal genome, which then decreases back to baseline (358). This spike in 5hmC is not seen in the maternal genome because it is passively demethylated. One suggested theory for the asymmetry in DNA demethylation between the paternal and maternal genome is that the two genomes attempt to achieve equal 5mC levels, considering the sperm has significantly higher 5mC content compared to oocytes before fertilization (358). The methylation content levels reach their lowest point at blastocyst formation. Overall, this process is called epigenetic programming (Figure 2.12). Maintenance CpG DNMT1 uses hemimethylated sites to ensure DNA methylation patterns, whereas de novo
CpG DNMT3a and 3b do not require preexisting methylation and therefore, establishes a new DNA methylation pattern (364). The new DNA methylation pattern is considered stable postnatally but it can be modified by factors such as aging, cancer, certain drugs (e.g. Decitabine) or the environment including diet. These factors can cause changes in the patterns of DNA methylation at the global or gene-specific level later in life and potentially influence adverse health outcomes and disease susceptibility. Aberrant or disturbed epigenetic programming owing to metabolic, hormonal, and/or nutritional changes in the intrauterine environment, which may influence disease susceptibility and health outcomes in the offspring in adulthood, is one of the proposed mechanisms associated with the Developmental Origins of Health and Disease (DOHaD) (39).

**Figure 2.12. Epigenetic programming.** After fertilization, active demethylation of the paternal and passive demethylation of the maternal genome occurs. Simultaneously, there is a rapid increase in 5-hydroxymethylcytosine (5hmC) levels in the paternal genome only. 5-methylcytosine (5mC) and 5hmC levels reach their lowest level at implantation. Levels of 5mC and 5hmC then rise to program the new methylation pattern of the offspring. ESCs, embryonic stem cells; ICM, inner cell mass cells (cells forming the embryo); TE, trophoblast cells (cells forming the placenta). Reprinted with permission from Wu and Zhang 2014 (358).
2.6.4 DNA Methylation and Hydroxymethylation and Health

Environmental factors, including maternal diet, chemical exposures, and stress, during intrauterine and early postnatal life can affect lifelong health and risk of disease. This field of study, known as DOHaD, has been in existence for nearly 50 years. Examples of the DOHaD paradigm include a link between low birth weight and risk of death from ischemic heart disease in adult males from the British Hertfordshire Cohort (382) and maternal exposure to under-nutrition during the Dutch Winter Famine and increased metabolic and cardiovascular disease in the offspring (383-385). Furthermore, several human studies, including those from the Dutch Winter Famine (386) and the Overkalix Cohort in Sweden (387), have demonstrated transgenerational health effects in the grandchildren, underscoring the importance of determining modulators of long-term health. Since epigenetic patterns are established in utero, alterations in this dynamic process resulting from maternal environmental exposure has been proposed to be one of the underpinning mechanisms in the DOHaD hypothesis and recent developments have generated tremendous interest in this area of research (39, 40, 388).

At present, the study of maternal and postnatal nutrition and its effects on DNA methylation patterns as a predictor of health and risk of disease in the offspring has become a rapidly emerging focus of research in this field. A cornerstone of DOHaD is the predictive adaptive response (PAR) hypothesis (389), which suggests if the developing organism can predict and adapt to the postnatal environment based on cues from the prenatal environment, it will be better suited for the future environment (matched environments). If a mismatch occurs, the risk of developing adverse effects in later life increases. Many of the mechanisms underlying PAR involve epigenetic processes (390). Some studies have supported the PAR hypothesis (82, 391),
while others have not (81). For example, the obesogenic phenotype of offspring born to dams fed a diet containing 10X the recommended vitamin levels (including 20 mg folic acid/kg diet) was prevented by feeding the offspring the same high multivitamin diet, or a diet high in folic acid alone (20 mg folic acid/kg diet) (82). In contrast, in another rodent study, a high folate diet (20 mg folic acid/kg diet) during pregnancy reduced body weight gain and was associated with smaller femoral area in the female offspring. However, matching the high folate dam diet with a high folate diet (20 mg folic acid/kg diet) in female pups did not correct the reduced body weight of the high folate dam diet, yet a mismatch of maternal and pup diet resulted in lower femoral peak load strength and stiffness (81). Discrepancies in the influence of maternal versus postnatal diet may be modulated by critical periods of growth and development of the target organ given each tissue has a critical developmental period, which may occur in utero, postnatally, or both (392).

Studies examining the effects of alterations in the intrauterine one-carbon nutrition milieu resulting from changes in maternal intake of one-carbon nutrients on DNA methylation in the developing fetus have begun to emerge. For example, several preliminary rodent studies have shown that maternal folic acid supplementation during pregnancy can modify the offspring’s methylome with subsequent changes in phenotype and disease susceptibility in adulthood (393, 394), which will be described in more detail in 2.6.5.1.
2.6.5 One-carbon nutrients and DNA methylation and hydroxymethylation

The biological mechanistic link between one-carbon nutrients (folate, vitamins B₁₂ and B₆, choline and betaine) and DNA methylation patterns is highlighted in the one-carbon metabolic pathway (Figure 2.3). One-carbon nutrients are metabolically related in the generation of SAM. A crucial enzyme involved in the generation of SAM regeneration is MS (395). MS requires vitamin B₁₂ as a coenzyme for optimal function. MS transfers a methyl group from 5-methylTHF, the main form of circulating folate, to homocysteine in remethylation of homocysteine to methionine. Homocysteine can also be remethylated to methionine by BHMT, using betaine as the methyl donor group, which is metabolized to dimethylglycine. Choline, which is the precursor of betaine, is formed from PC, the endogenous form of choline. De novo synthesis of PC occurs through the conversion of PE via PEMT. Methionine is converted to SAM by methionine adenosyltransferase (MAT). SAM is the universal methyl group donor for over 100 biological methylations reactions including DNA methylation (1). To avoid buildup of toxic levels of homocysteine, vitamin B₆ aids in the clearance of homocysteine to cysteine via the transsulfuration pathway mediated by CβS. Vitamin B₆ is also involved in the conversion of serine to glycine by acting as a coenzyme for SHMT, which catalyzes the conversion of THF to 5,10-methyleneTHF. Given that one-carbon nutrients can contribute to the generation of SAM, deficiencies/excesses or imbalances of these nutrients can potentially influence DNA methylation.

One-carbon nutrients, either individually or in combination, have the potential to modulate the
methyl donor “pool” available for DNA methylation reactions. Results from animal studies have suggested that deficiency and supplemental levels of folate on DNA methylation are gene and site-specific and depend on cell type, target organ, and stage of transformation as well as on the timing, degree, and duration of intervention (393, 394, 396-401). In humans, metabolic studies demonstrated that controlled folate depletion reduces global DNA methylation in leukocytes of older individuals (402). However, there are no conclusive data suggesting that folate deficiency induces significant global DNA hypomethylation in tissues such as the colorectum (395). In contrast, folic acid supplementation, even at modest supplemental levels, appears to be able to increase global DNA methylation, albeit inconsistently, in circulating peripheral mononuclear cells and in certain tissues such as the colorectum (395). The majority of observational studies have described a direct relationship between dietary and blood levels of folate and global DNA methylation in both lymphocytes and colonic tissues (395). This positive association is more consistent in individuals with colorectal adenomas, adenocarcinomas, or previously resected neoplastic tumors as well as in those at a greater risk of health complications compared with normal subjects (395). Taken together, these studies justify the need to investigate the effects of one-carbon nutrients in periods of growth and development that are particularly vulnerable to environmental dietary exposures, specifically intrauterine development.

Although indirect mechanisms may be plausible, there is currently no known direct mechanism by which one-carbon nutrients can modulate DNA hydroxymethylation. Nonetheless, understanding the important role of one-carbon nutrients on DNA methylation cannot be complete without investigating the effects of these nutrients on DNA hydroxymethylation.
2.6.5.1 Effects of maternal one-carbon nutrient environment on DNA methylation in the animal offspring

Studies using viable yellow agouti mice have demonstrated that maternal dietary supplementation of folic acid, vitamin B₁₂, choline and betaine can permanently alter the coat color of the offspring via increased CpG methylation in the promoter region of the agouti gene (393, 403) (Table 2.11). Similarly, the same methyl group-rich diet has been shown to significantly reduce the proportion of progeny with a kinked tail phenotype and this was paralleled to a higher degree of CpG methylation in the promoter region of the AxinFused gene (394). In the agouti mouse model, one-carbon nutrients have also been shown to interact with other environmental exposures during the intrauterine period to modulate DNA methylation in the developing offspring. For example, bisphenol A is an estrogenic xenobiotic chemical used in the manufacturing of polycarbonate plastics and is associated with higher body weight, increased risk of cancer, and other chronic health conditions (404). Exposure to this chemical in utero has been shown to change the coat color of agouti mice by decreasing CpG methylation of the agouti gene (399). Maternal methyl donor supplementation including folic acid has been shown to reverse the epigenetic and phenotypic effects of bisphenol A in the offspring (399). In rats, promoter DNA methylation of the Pparγ and Gr genes have been observed to be significantly lower, by 20% and 22.8%, respectively, in offspring from dams fed a protein restricted diet compared with a control diet during pregnancy, which resulted in increased expression of the Pparγ and Gr proteins (396). Maternal folic acid supplementation prevented these changes (396).

Investigation of the effect of maternal folic acid intake on DNA methylation changes in the offspring has garnered significant interest in this field of research (Table 2.11). In mice
heterozygous for the folate binding protein gene (Folbp1+/−), daily administration of folinic acid (5-formylTHF) by gavage during the periconceptional period until day 15.5 of gestation significantly decreased global DNA methylation in both the liver and brain of the offspring (405). In rats, global DNA methylation was 25% lower in the liver of pups from dams supplemented with folic acid during pregnancy and lactation than from dams on the control diet (p<0.001) (406). Furthermore, maternal folic acid supplementation significantly decreased DNA methylation in specific CpG sites of the promoter region of the Ppar-γ and ER-α genes (p < 0.05), in exons 6 and 7 of the p53 gene (p < 0.01) and in exon 15 of the Apc gene (p < 0.01) but not in the promoter region of the p16 gene in the pup liver (406). In other rodent studies, maternal folate deficiency and supplementation with other nutritional factors were not observed to affect offspring liver global methylation (407, 408). However, in hyperhomocysteinemic rats, maternal dietary folate deficiency was significantly associated with placenta global DNA hypomethylation (400). In addition, significant positive correlations between placental global DNA methylation and folate concentrations in the placenta, plasma, and liver were reported (400). Kulkarni et al. also investigated the effects of maternal folic acid supplementation with or without vitamin B12 on placental global DNA methylation in rats and reported that maternal folic acid supplementation (4x the basal requirement) in the absence of vitamin B12 results in global DNA hypomethylation, suggesting that the ratio of folic acid and vitamin B12, rather than the actual supplemental level, may have an important role in determining global DNA methylation patterns (401). More recently, a study examining both maternal and postweaning folate status on global DNA methylation in the small intestine of adult mice offspring observed that maternal folate depletion (1/5 of the control diet) during pregnancy and lactation was associated with DNA hypomethylation compared to control (2 mg/kg diet) and supplemented (4x control)
groups, regardless of folic acid content in the postweaning diet (409). In the sheep model, maternal periconceptional folate and vitamin B₁₂ restriction led to aberrant DNA methylation patterns in 4% of the 1400 CpG islands examined in the offspring (398). These DNA methylation changes were associated with increased adiposity, insulin resistance, altered immune function, and high blood pressure in the adult male offspring (398).

Experimental animal tumorigenesis models have shown that maternal dietary folate manipulation can modify both DNA methylation patterns and the risk of cancer in the developing offspring. Maternal folic acid supplementation, equivalent to ~1000 µg folic acid/d in humans, provided in utero and during lactation significantly decreased mammary global DNA methylation by 7%, and increased the risk of mammary tumors in the offspring (79). In contrast, maternal folic acid supplementation at the same level and duration significantly increased colorectal global DNA methylation by 3%, and reduced the odds of developing colorectal tumors in the offspring (80). Similarly, in the Apc^{1638N} spontaneous mouse model of CRC, offspring of vitamin B₂, B₆, B₁₂ and folic acid supplemented mothers displayed a mild degree of global DNA hypomethylation in the small intestine mucosa and decreased tumor occurrence (410). This suggests that the effect of maternal one-carbon nutrient supplementation on cancer risk in the offspring may be organ specific and the outcome may be mediated in part by changes in global DNA methylation.

Collectively, evidence from animal studies provide proof-of-principle that DNA methylation of the offspring can be significantly modulated by intrauterine exposure to varying levels of one-carbon nutrients with consequent permanent changes in phenotypes and other functional outcomes. However, extrapolation to humans is still limited. For example, the nutrient sensitive
gene loci $A^{vy}$ and $Axin^{FU}$ are not present in the human genome (308). The majority of studies were conducted in rodent models, which give birth to relatively immature offspring (411), which may have implications on their susceptibility to the postnatal environment compared to humans. Furthermore, the majority of animal models employ inbred strains (308), while humans are both genetically and epigenetically diverse. Nonetheless, data from animal models provide valuable insight because they have shorter gestational periods, a distinct visual marker (coat color in the agouti mice and tail kink in the AxinFused mice), specific DNA methylation changes that correspond to phenotypic changes, and are prone to human diseases (diabetes, obesity, and cancer) in certain models (308).

Table 2.11. Summary of in utero and perinatal one-carbon nutrient supplementation on DNA methylation in the animal offspring

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Species</th>
<th>One-carbon nutrient source &amp; levels</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finnell et al. 2002 (405)</td>
<td>Mice $Folbp1^{+/−}$</td>
<td>25 mg/kg/d Folinic acid by gavage</td>
<td>2 weeks prior to mating to gestational day 15.5</td>
<td>Liver</td>
<td>Genomic</td>
<td>~4-fold decrease (P&lt;0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain</td>
<td>Periconceptional</td>
<td></td>
</tr>
<tr>
<td>Waterland et al. 2003 (403)</td>
<td>Mice $A^{vy/a}$</td>
<td>1) 5 g choline, 5 g betaine, 0.5 mg folic acid, 0.5 mg vit. $B_{12}$</td>
<td>2 weeks prior to and throughout pregnancy and lactation</td>
<td>Tail tip</td>
<td>Seven CpG sites in Pseudoexon 1A</td>
<td>CpG methylation increase (P=0.005) leading to coat color change (P=0.001)</td>
</tr>
<tr>
<td></td>
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<td>2) 2.5 g choline, 2.5 g betaine, 2.5 mg folic acid, 0.25 mg vit. $B_{12}$</td>
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<tr>
<td>Study (reference)</td>
<td>Species</td>
<td>One-carbon nutrient source &amp; levels</td>
<td>Duration</td>
<td>Tissue</td>
<td>DNA methylation</td>
<td>Effect</td>
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</tbody>
</table>
| Waterland et al. 2006  | Mice    | 1) 5 g choline, 5 g betaine, 5 mg folic acid, 0.5 mg vitamin B12  
2) 2.5 g choline, 2.5 g betaine, 2.5 mg folic acid, 0.25 mg vitamin B12 | 2 weeks prior to and throughout pregnancy and lactation | Tail tip | Axin<sup>Fu</sup> gene | Increased CpG methylation (P=0.009) leading to less tail kinks (P<0.0001)  
Increased CpG methylation (P=0.05) leading to less tail kinks (P<0.0001) |
| Sinclair et al. 2007   | Sheep   | Vitamin B<sub>12</sub> & folate deficient vs. control | 8 weeks prior to 6 days after conception | Liver  | 1400 CpG sites | 4% of CpG sites had altered status (P < 0.001) |
| Maloney et al. 2007    | Rats    | 1) Control  
2) Folate -/-  
3) Folate -/- & low methionine  
4) Folate -/- & low choline  
5) Folate -/- & low methionine & low choline | 2 weeks prior to mating to D21 of gestation | Liver  | Genomic         | No change |
| Engeham et al. 2009    | Rats    | 1) Control: 18% casein, 1mg/kg folic acid  
2) Control with folate: 18% casein, 5mg/kg folic acid  
3) Low protein: 9% casein, 1mg/kg folic acid  
4) Low protein with folate: 9% casein, 5mg/kg folic acid | Pregnancy | Liver  | Genomic         | No change |
<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Species</th>
<th>One-carbon nutrient source &amp; levels</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
</table>
| Kim *et al.* 2009 (400) | Rats | 1) 8 mg/kg folic acid  
2) 8 mg/kg folic acid & 0.3% Hcy  
3) 0.3% Hcy | 4 wks prior to d20 of pregnancy | Placenta | Genomic correlation with  
Plasma folate  
Liver folate  
Placenta folate | $r = 0.752\, (P = 0.0003)$  
$r = 0.700\, (P = 0.0012)$  
$r = 0.819\, (P < 0.0001)$ |
| Kulkarni *et al.* 2011 (401) | Rats | 8 mg/kg folic acid & B$_{12}$ -/- vs. 2 mg/kg folic acid & B$_{12}$ -/- | Pregnancy | Placenta | Genomic | Decreased ($P < 0.05$) |
| McKay *et al.* 2011 (409) | Mice | 0.4 mg/kg folic acid vs. 2 mg/kg folic acid or 8 mg/kg folic acid | Mating, pregnancy and lactation | Small intestine | Genomic | Decreased ($P = 0.009$)  
Decreased ($P = 0.006$) |
| Ly *et al.* 2011 (79) | Rats | 5 mg/kg folic acid vs. 2 mg/kg folic acid | 3 weeks prior to mating through pregnancy and lactation | Mammary | Genomic | 7% decrease  
($P = 0.03$) |
| Sie *et al.* 2011 (80) | Rats | 5 mg /kg folic acid vs. 2 mg/kg folic acid | 3 weeks prior to mating through pregnancy and lactation | Colorectum | Genomic | 3% increase  
($P = 0.007$) |
| Ciappio *et al.* 2011 (410) | Mice $Apc^{1638N}$ | Vitamin B supplemented  
(8 mg/kg folic acid) vs. control  
(2 mg/kg folic acid) | 4 weeks prior to mating through pregnancy and lactation | Small intestine mucosa | Genomic | ~3% decrease  
($P = 0.07$) |
<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>Species</th>
<th>One-carbon nutrient source &amp; levels</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sie et al. 2013 (406)</td>
<td>Rats</td>
<td>5 mg/kg folic acid vs. 2 mg/kg folic acid</td>
<td>3 weeks prior to mating through pregnancy and lactation</td>
<td>Liver</td>
<td>Genomic</td>
<td>25% decrease at weaning (P&lt;0.001) At weaning, CpG methylation decreased in all but p16</td>
</tr>
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<td></td>
<td></td>
<td>Pparγ</td>
<td>P &lt; 0.05</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p53</td>
<td>exons 6, 7 (P &lt; 0.01)</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Apc</td>
<td>exon 15 (P &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p16</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ERα</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

More recently, the question of whether or not mothers themselves may be susceptible to changes in DNA methylation as a result of modified folate nutrition during pregnancy has been raised. In female mice fed folate-adequate (control) and folate-deficient (1/5 of control) diets prior to mating and during pregnancy and lactation, post-partum DNA methylation of Igf2 DMR1 and Slc39a4 at specific CpG sites was significantly lower in folate-deficient mice (409).

Interestingly, for Igf2 DMR2, a significant interaction between dietary folate and tissue DNA methylation was observed where DNA methylation was lower in blood and higher in liver in low-folate dams (409).
2.6.5.2 Effects of maternal one-carbon nutrient environment on DNA methylation in the human offspring

Given the association between folate deficiency and the risk of NTDs, several human studies have investigated whether aberrant DNA methylation patterns can be an underlying mechanism of NTDs and other congenital defects. Two case-control studies have examined the relationship between maternal folate status and global DNA methylation of neural tissue from NTD affected human fetuses (412, 413). While both studies reported lower maternal circulating folate concentrations and global brain DNA hypomethylation in fetuses affected with NTD compared with controls, only the study by Chang et al. observed a significant correlation between the DNA methylation changes and folate status (413). Both studies suggest that folate inadequacy may interfere with one-carbon metabolism and hence, DNA methylation, which may affect normal fetal development.

A preliminary prospective study in the United Kingdom found an inverse correlation between cord plasma homocysteine concentrations and global DNA methylation using methylation of long interspersed nucleotide element (LINE-1) sequences as a surrogate in cord lymphocytes in the offspring of 24 pregnant women (414) (Table 2.12). Although the results of this study are consistent with the biological functions of folate, no significant associations between maternal folic acid use and cord blood folate concentrations and global lymphocyte DNA methylation were observed. Interestingly, however, an association between global lymphocyte DNA methylation and fetal birth weight was identified in this study (414). Further interrogation of specific CpG loci associated with selected candidate genes in a subset of the same cohort found
that CpG DNA methylation patterns were also significantly correlated with plasma homocysteine, LINE-1 DNA methylation and birth weight centile (415).

A growing number of studies have investigated the effect of maternal folate exposure on CpG methylation of the imprinted insulin-like growth factor 2 (IGF2) gene, important for growth and development. However, the results have been inconsistent. A cross-sectional examination of a population unexposed to folic acid supplements before and during pregnancy revealed no significant associations between promoter DNA methylation of the IGF2 gene in cord blood and serum folate concentrations in either mother’s or cord blood (416). However, in an observational study conducted in The Netherlands, periconceptional maternal folic acid use of 400 µg/day significantly increased, by 4.5 %, DNA methylation of the IGF2 differentially methylated region (IGF2 DMR) in whole blood derived from children at 17 months of age (417). An independent inverse association was also observed between IGF2 DMR methylation and birth weight. More recently, the North American Newborn Epigenetic STudy (NEST) also evaluated the exposure of maternal folic acid supplementation before and during pregnancy on the DNA methylation profile of two DMRs known to regulate IGF2 expression in cord blood leukocytes (418). No evidence for DNA methylation differences at the IGF2 DMR were found while DNA methylation levels at the second H19 DMR were significantly lower among folic acid users compared to non-users (418). IGF2 DMR DNA hypomethylation and H19 DMR DNA hypermethylation have both been independently associated with increased IGF2 transcriptional activity and loss of imprinting, and has been observed in several human malignancies (418). However, data from the Generation R Study in The Netherlands found no association between maternal folic acid supplementation and DNA methylation in 11 regions of seven genes (NR3C1, DRD4, 5-HTT, IGF2 DMR, H19, KCNQ1OT1, and MTHFR) (419). While some of the
aforementioned studies have reported shifts in DNA methylation changes of the *IGF2* and *H19* DMRs with folic acid supplementation, the functional outcomes of these DNA methylation changes have not been clearly established. The dose and timing of folic acid appear to play a significant role in modulating DNA methylation. For example, Haggarty *et al.* reported a positive association with *IGF2* DNA methylation and inverse association with *PEG3* and LINE-1 DNA methylation with maternal folic acid supplementation after the first trimester (>12 weeks gestation), but not with preconceptional or first trimester supplementation (420).

In a French population, folic acid supplementation or the combination of folate, vitamin B<sub>12</sub> and vitamin B<sub>6</sub> intake before or during pregnancy was not associated with DNA methylation of the *PLAGL1* DMR, a tumor suppressor gene encoding zinc finger protein, in cord blood leukocytes (421). On the contrary, an inverse relationship between increasing maternal RBC folate concentrations and *PLAGL1* DNA methylation in cord leukocytes was found in an American mother-child cohort (422).

In addition to maternal folate intake and folic acid supplementation, maternal vitamin B<sub>12</sub>, choline and betaine intake has been linked to changes in DNA methylation content in human offspring. Increased maternal serum vitamin B<sub>12</sub> during pregnancy was associated with a decrease in global DNA methylation in cord blood (423). In a folate-replete American population, Boeke *et al.* did not find a relationship between maternal intakes of folate, vitamin B<sub>12</sub>, choline or betaine with global LINE-1 DNA methylation in cord leukocytes. After stratifying between male and female offspring, however, higher maternal choline and betaine intake was associated with lower cord LINE-1 DNA methylation in male offspring (424).
Table 2.12. Summary of in utero and perinatal one-carbon nutrient status on DNA methylation in the human offspring

<table>
<thead>
<tr>
<th>Study (reference)</th>
<th>One-carbon nutrient source &amp; levels</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fryer et al. 2009 (414)</td>
<td>Folic acid supplement, 400 µg/day</td>
<td>Pregnancy</td>
<td>Cord blood lymphocytes</td>
<td>Global ((LINE-1))</td>
<td>(r = 0.364\ (P = 0.08))</td>
</tr>
<tr>
<td></td>
<td>Cord serum folate, 15.8 µmol/l</td>
<td></td>
<td></td>
<td></td>
<td>(r = 0.209\ (P &gt; 0.05))</td>
</tr>
<tr>
<td></td>
<td>Cord Hcy, 10.8 µmol/l</td>
<td></td>
<td></td>
<td></td>
<td>(r = -0.688\ (P =0.001))</td>
</tr>
<tr>
<td>Steegers-Theunissen et al. 2009 (417)</td>
<td>Folic acid supplement, 400 µg/day</td>
<td>Periconceptional</td>
<td>Whole blood at 17 mo old</td>
<td>(IGF2)</td>
<td>4.5% higher, (P = 0.014)</td>
</tr>
<tr>
<td>Fryer et al. 2011 (415)</td>
<td>Folic acid supplement, 400 µg/day</td>
<td>Pregnancy</td>
<td>Cord blood lymphocytes</td>
<td>27,578 CpG loci associated with 14,496 genes</td>
<td>No association with supplementation</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
<td>CpG methylation correlated with</td>
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<td></td>
<td>plasma Hcy (P=0.04),</td>
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<td></td>
<td></td>
<td></td>
<td>(LINE-1) methylation (P=0.03), birth weight percentile (P=0.02)</td>
</tr>
<tr>
<td>Ba et al. 2011 (416)</td>
<td>Cord serum folate, 7.29 ng/ml</td>
<td>NA (Observational design)</td>
<td>Cord blood mononuclear cells</td>
<td>(IGF2) promoter2 (IGF2) promoter3</td>
<td>No association</td>
</tr>
<tr>
<td></td>
<td>Maternal serum folate, 2.29 ng/ml</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hoyo et al. 2011 (418)</td>
<td>Folic acid users vs. non-users before or during pregnancy</td>
<td>NA (Observational design)</td>
<td>Cord blood leukocytes</td>
<td>(IGF2) DMR (H19) DMR</td>
<td>No association</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.8% decrease (P = 0.03) before pregnancy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.9% decrease (P = 0.04) during pregnancy</td>
</tr>
<tr>
<td>Study (reference)</td>
<td>One-carbon nutrient source &amp; levels</td>
<td>Duration</td>
<td>Tissue</td>
<td>DNA methylation</td>
<td>Effect</td>
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<tr>
<td>Boeke et al. 2012 (424)</td>
<td>Dietary intakes of folic acid, vitamin B₃₂, choline, betaine</td>
<td>Pregnancy</td>
<td>Cord blood leukocytes</td>
<td>Global (LINE-1)</td>
<td>No association</td>
</tr>
<tr>
<td>McKay et al. 2012 (423)</td>
<td>Maternal RBC folate, 379 ng/ml Maternal vitamin B₁₂, 283 pg/ml</td>
<td>NA (Observational design)</td>
<td>Cord blood</td>
<td>Global (LUMA)</td>
<td>Maternal vitamin B₁₂ inversely associated with cord DNA methylation (P=0.04)</td>
</tr>
<tr>
<td>Haggarty et al. 2013 (420)</td>
<td>Folic acid supplement users</td>
<td>Before and after 12 weeks gestation</td>
<td>Cord blood</td>
<td>Global (LINE-1) IGF2 PEG3 SNRPN</td>
<td>Supplementation &gt;12 weeks gestation associated with -0.3% (P= 0.03) +0.7% (P= 0.04) -0.5% (P= 0.02) No association</td>
</tr>
<tr>
<td>van Mil et al. 2014 (419)</td>
<td>Folic acid supplement use</td>
<td>NA (Observational design)</td>
<td>Cord blood leukocytes</td>
<td>11 regions of seven genes NR3C1, DRD4, 5-HTT, IGF2 DMR, H19, KCNQ1OT1, MTHFR</td>
<td>No association with maternal homocysteine and folate concentrations, folic acid supplement use, and the MTHFR genotype</td>
</tr>
<tr>
<td>Azzi et al. 2014 (421)</td>
<td>Supplementation with folic acid alone, or the combo of folic acid, vit B₆ &amp; B₁₂</td>
<td>Before or during pregnancy</td>
<td>Cord blood leukocytes</td>
<td>PLAGLI</td>
<td>No association</td>
</tr>
<tr>
<td>Study (reference)</td>
<td>One-carbon nutrient source &amp; levels</td>
<td>Duration</td>
<td>Tissue</td>
<td>DNA methylation</td>
<td>Effect</td>
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</tbody>
</table>
| Hoye *et al.* 2014 (422) | Quartiles of RBC folate concentrations | NA (Observational design) | Cord blood leukocytes | *IGF2*  
*H19*  
*DLK1/MEG3*  
*PEG1/MEST*  
*PEG3*  
*PEG10/SGCE*  
*PLAGL1*  
*NNAT* | Q2 < Q1 (P= 0.004)  
No association  
Q4 > Q1 (P= 0.001)  
No association  
Q2 > Q1 (P= 0.03)  
No association  
Q3 < Q1 (P= 0.01)  
No association |

Although it is difficult to compare directly the outcomes of the studies reviewed above due to inherent differences in study design, research models utilized, and techniques used to interrogate DNA methylation as well as the populations’ baseline one-carbon nutrient status and/or supplementation habits, several lines of evidence support the conjecture that DNA methylation patterns of the developing fetus can be significantly modulated by intrauterine environmental exposures, including one-carbon nutrients. Furthermore, the changes in DNA methylation status may be associated with developmental changes and permanent alterations in phenotype with potential health consequences in later adult life. Whether or not one-carbon nutrient-mediated epigenetic changes *in utero*, however, can affect the risk of chronic disease development in adulthood remains unclear.
Chapter 3: Rationale, Objectives and Hypothesis

With the introduction of folic acid fortification in 1998, folic acid intake has significantly increased over the past 15 years in Canada. This combined with widespread folic acid supplemental use in women of childbearing age before and during pregnancy inadvertently expose the developing fetus to high levels of folic acid. Increased intakes of dietary and supplemental folic acid have led to detectable levels of UMFA in the general population as well as in pregnant women and their infants, the consequences of which are currently unknown. Furthermore, intakes of other one-carbon nutrients, including vitamins B₆ and B₁₂ and choline, have not been well characterized in pregnancy. The status of these one-carbon nutrients during pregnancy may have individual or synergistic effects on fetal development and health outcomes of the offspring. One-carbon nutrients have a potential to modulate DNA methylation because they are integrally related in the one-carbon metabolic pathway leading to the generation of SAM, the universal methyl group donor for over 100 biological methylation reactions including DNA methylation. DNA methylation is an epigenetic determinant in gene expression and genomic stability, thereby playing a critical role in human health and disease susceptibility. DNA methylation is programmed during embryogenesis in order to establish a unique DNA methylation pattern, which is maintained postnatally. Aberrant patterns or dysregulation of DNA methylation programming during embryogenesis and early life have been posited to influence disease susceptibility of the offspring later in life. Hormonal, metabolic, and nutritional intrauterine environment plays a critical role in DNA programming. In particular, previous animal studies have shown that maternal status of one-carbon nutrients can modulate DNA methylation in the offspring, leading to permanent phenotypic changes and disease susceptibility. Additionally, human studies have also shown maternal diet, including one-carbon nutrients, can
alter DNA methylation patterns in the offspring; however, the results have not been consistent and these studies are limited in scope.

Given these considerations, the overall research question is: can maternal intake and blood concentrations of one-carbon nutrients have an epigenetic effect in the human offspring?

I characterized maternal and cord blood concentrations of one-carbon nutrients, folate, vitamins B₆ and B₁₂, and choline, which are critical for DNA methylation and fetal development and determined the potential modifying effects of fetal genetic variants in one-carbon metabolism on cord blood concentrations in a large cohort of Canadian pregnant women. In this thesis, the results for folate and vitamin B₆ are presented and the results for vitamin B₁₂ and choline have been presented elsewhere. I then examined the effects of maternal status of folate, vitamin B₁₂, vitamin B₆, choline and betaine on DNA methylation and hydroxymethylation in cord blood MNCs and the potential modifying effects of fetal genetic variants in one-carbon metabolism. The studies included in this thesis will provide significant information regarding the maternal status of one-carbon nutrients during pregnancy and the effects of maternal one-carbon nutrients on DNA methylation and hydroxymethylation of the developing fetus. These studies will provide a framework for future research to elucidate the functional ramifications and clinical implications of maternal one-carbon nutrient status on the health of human offspring.

Specific Research Questions

1) What are the concentrations of folate (including UMFA), vitamin B₆, and homocysteine in a cohort of Canadian pregnant women during early and late pregnancy and in the cord blood?
2) Do maternal concentrations of folate, vitamin B₆, and homocysteine predict the concentrations of folate, PLP, and homocysteine, respectively, in the cord blood?

3) Do maternal diet and fetal genetic variants influence maternal and cord concentrations of these biomarkers, respectively?

4) Can maternal intake and blood concentrations of one-carbon nutrients have an effect on DNA methylation and hydroxymethylation content in human cord blood MNCs?

Hypotheses

I hypothesize that:

1) maternal dietary intakes of folate and vitamin B₆ will be positively associated with concentrations of these nutrients in maternal and fetal compartments

2) fetal genetic variants will alter cord concentrations of folate, PLP, and homocysteine

3) concentrations of maternal one-carbon nutrients (serum and RBC folate, PLP, vitamin B₁₂, choline, betaine) and their metabolites and biomarkers (homocysteine, MMA, DMG, and TMAO) will be correlated with 5mC and 5hmC content cord blood MNCs

Objectives

1) To determine and characterize maternal and cord blood concentrations of folate and vitamin B₆ in a cohort of Canadian pregnant women and their newborn infants

2) To assess the association between maternal and cord blood concentrations of folate and vitamin B₆

3) To assess the association of maternal folate and vitamin B₆ intakes with maternal and cord blood concentrations of these nutrients
4) To examine the effect of fetal genetic polymorphisms of one-carbon metabolism on cord blood concentrations of folate, vitamin B₆, and homocysteine

5) To determine whether or not maternal blood concentrations of folate, vitamins B₆ and B₁₂, choline and betaine during pregnancy influence global DNA methylation and hydroxymethylation in cord blood MNCs.
Chapter 4: Study design, subjects, baseline and infant characteristics, and dietary and supplemental intakes of one-carbon nutrients in the PREFORM study


4.1 Overall study design

The PREFORM Study was a prospective observational study involving a Canadian mother-child cohort consisting of three study visits throughout pregnancy and childbirth. All women were recruited from St Michael’s Hospital (SMH), a tertiary teaching hospital fully affiliated with the University of Toronto. SMH is located in downtown Toronto, ON and services approximately 3000 live births per year. This study protocol received ethical approval by the Research Ethics Board at St. Michael’s Hospital (REB 10-246). This study is registered at www.clinicaltrials.gov (ClinicalTrials.gov identifier: NCT02244684).

Inclusion criteria were women between 18-45 years old with a singleton pregnancy with no intention to bank umbilical cord blood and did not have a condition that interfered with folate metabolism and absorption. This includes celiac disease, inflammatory bowel disease including
Crohn’s disease and ulcerative colitis, gastric bypass surgery or use of antifolate medications. If a participant was found to be folate deficient (serum folate <6.8 nmol/l), she was to be notified and excluded from the study. However, no women were excluded from the study for this reason.

**Sample size rationale**

A sample size of 260 mother-child pairs was calculated to be more than adequate to provide 90% power to detect a correlation of 0.2 or larger between serum folate concentrations and the amount of global DNA methylation in the umbilical cord blood MNCs (414). However, this study was not powered to detect a relationship with other one-carbon nutrients and DNA methylation. This calculation took into consideration the anticipated unequal number of mothers who would report periconceptional (~60%) and prenatal (~90%) folic acid-containing supplement use (189). Based on our prior experience with clinical studies involving women and on results reported in the literature, we anticipated an overall dropout rate of approximately 15%. Thus, in order to meet our required sample size, we planned on recruiting 300 mother-child pairs.

PREFORM Study visits are outlined in **Figure 4.1**.
Figure 4.1. PREFORM Study visits.

i. Study Visit 1 (Baseline, 12-16 weeks gestation)

Consenting participants completed a Demographic and Health Questionnaire (DHQ) which consisted of sections of the Canadian Community Health Survey (CCHS) cycle 2.2 (425) and the 2005 modified Block Food frequency questionnaire (FFQ) (NutritionQuest, Berkeley, CA). The CCHS is a standardized questionnaire which has been administered in populations across Canada and collects information on maternal age, race-ethnicity, family income, number of dependents, highest level of education, previous pregnancies, number of live births, smoking and alcohol history and anthropometric data including current height and weight, diagnosis of seizure disorder requiring daily drug use and diagnosis of diabetes are documented. The 110-food item modified FFQ captures dietary intake during the periconceptional period (previous 3 months)
and includes questions about vitamin and supplement use and has previously been validated in a pregnant population (426). The 30-minute FFQ was either self-administered online or completed in person by interview to identify the frequency of dietary intake of food and beverage items. The FFQ was accompanied by images to depict portion sizes to increase accuracy of food quantity. Nutrient intakes were computed by NutritionQuest. For these computations, NutritionQuest used the 2001–2002 NHANES dietary recall data and nutrient composition data primarily from version 1.0 of the USDA Food and Nutrient Database for Dietary Studies (427). Because different fortification regulations exist for ready-to-eat breakfast cereals and rice in Canada and the US, the folic acid, vitamin B₆, and vitamin B₁₂ composition values were modified for these food items using values from the Canadian Nutrient File (428). Choline and betaine were not assessed in the 2001–2002 cycle of NHANES; hence, the choline and betaine values for each FFQ food item in the present study were derived from the “2004 and 2008 USDA Databases for the Choline Content of Common Foods” (429). The Block FFQ has been validated in a number of populations including pregnant women and in a sample of Ontario women of reproductive age (430, 431) and was shown to be a moderately accurate estimate of B vitamin intake (430). Specifically, the median deattenuated Pearson correlation coefficients between the Block FFQ and two 24-h recalls were reported to be 0.76 for folate, 0.70 for vitamin B₆, and 0.65 for vitamin B₁₂ from supplements and food (430).

Maternal blood was routinely drawn at 12 and 16 weeks gestation for the integrated prenatal screening (IPS) 1 and 2, respectively. An additional 19ml maternal blood was drawn for the PREFORM study at IPS1 or IPS2. All blood was collected using vacutainers with EDTA (for plasma and RBC analysis) or serum separator tubes with silica clot activator (serum analysis) (Becton Dickinson, Franklin Lakes, New Jersey) by SMH staff. Serum and RBC folate, plasma
homocysteine and serum vitamin B$_{12}$ were measured by SMH core laboratory facilities. For each participant, two 2ml plasma samples were aliquoted and stored at -80°C to establish the biorepository.

**ii. Study Visit 2 (34-37 weeks gestation)**

The follow-up study visit occurred at the 34 or 36 week obstetrical appointment. Participants were asked to complete the same 110-item modified FFQ in person or online to capture dietary intakes in the previous three month period. Participants were also given a study identifier sheet, which they were asked to present to SMH Labor & delivery staff when admitted for childbirth. The study identifier sheet listed the participants subject ID number and instructions on blood collection procedures for the attending nurses and obstetrical staff.

**iii. Study visit 3 (Parturition, 37-41 weeks gestation)**

Upon hospital admission for childbirth, maternal blood was drawn for a routine CBC test as part of standard hospital procedures. Simultaneously, 19ml of maternal blood was collected and used to measure the same parameters tested at baseline (serum and RBC folate, plasma homocysteine and serum vitamin B$_{12}$). At parturition, up to 20ml of umbilical cord blood was collected immediately after the newborn is cut from the cord but prior to placental delivery. All blood was collected into vacutainers containing EDTA for analysis. For each participant, two 2ml plasma samples of both maternal and cord blood were aliquoted and stored at -80°C in biorepository. A minimum of 8ml of whole blood from the umbilical cord was used for mononuclear cell (MNC) extraction. DNA and RNA were isolated from MNCs to be used for DNA methylation and gene expression functional assays, respectively.
After parturition, data on maternal weight gain during pregnancy, diagnosis of gestational diabetes, changes in medical history, and mode of delivery was obtained from maternal medical records. Infant sex, birth weight, length, head circumference, gestational age, size for gestational age, and 1 and 5-minute Apgar scores were obtained from medical records. This information was used to characterize the study population and served as potential confounding variables in the data analysis.

4.1.1 Recruitment

Pregnant women referred to the study obstetricians (Department of Obstetrics and Gynecology at SMH) were screened and approached by research staff in the clinic at their first or second prenatal appointment (between 12 and 16 weeks of gestation). Between November 2010 and December 2011, study staff approached 67% (n=1315) of women identified as potential participants. Of these, women 906 met eligibility criteria and 368 (42%) women agreed to participate (Figure 4.2). Written informed consent was obtained from 368 women. 364 women completed at least the DHQ, the baseline questionnaire used to assess demographics and health status patterned after the Canadian Community Health Survey cycle 2.2. Three hundred thirty nine women completed the first FFQ. A total of 35 participants did not complete the late pregnancy study visit and of those remaining (n = 319), 305 completed the second FFQ. At the time of delivery, data was collected from 309 subjects and their newborn infants. 15% (55 women) withdrew with the most common reason being they transferred their care to another center (Figure 4.2). There were no differences in maternal age, BMI, dietary intakes of folate, vitamins B₆ and B₁₂, choline or betaine, supplemental intakes of folic acid, vitamin s B₆ and B₁₂,
or blood concentrations of serum and RBC folate, UMFA, PLP, vitamin B₁₂, choline, betaine, DMG, or TMAO in early pregnancy between the participants lost to follow up and those who delivered at SMH. However, plasma homocysteine in early pregnancy was significantly higher in women lost to follow up [mean (95%CI): 5.4 (5.0, 5.8 nmol/L)] compared to those who delivered at SMH [5.0 (4.9, 5.1 nmol/L) p=0.04]. 1315 pregnant women were approached to participate. 339 completed the first FFQ. A total of 35 participants did not complete the late pregnancy study visit and of those remaining (n = 319), 305 completed the second FFQ.

Figure 4.2: Flowchart of participants through study visits 1 and 2 and reasons for loss-to-follow-up.
Data and specimen collection occurred during three study visits from 12 weeks gestation to parturition (Figure 4.2). Final follow-up study visits, deliveries and data collection were completed in August 2012.

4.2 Analyses of blood biomarkers

Aliquots of maternal and cord plasma were later used to analyze PLP, MMA, total choline, betaine, and other metabolites. A complete list of blood measurements and the assays used is listed in Table 4.1.

Table 4.1. PREFORM Study blood measurements in maternal and umbilical cord blood.

<table>
<thead>
<tr>
<th>Maternal blood</th>
<th>Umbilical cord blood</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate</td>
<td>Serum folate</td>
<td>Access Folate System (immunoassay)</td>
</tr>
<tr>
<td>RBC folate</td>
<td>RBC folate</td>
<td>Elecsys Folate Assay (immunoassay)</td>
</tr>
<tr>
<td>Plasma unmetabolized FA (UMFA)</td>
<td>Plasma UMFA</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>Plasma homocysteine</td>
<td>Plasma homocysteine</td>
<td>Synchron LX20/DxC System (immunoassay)</td>
</tr>
<tr>
<td>Serum B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Serum B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Access competitive-binding immunoenzymatic assay</td>
</tr>
<tr>
<td>Plasma Methylmalonic acid (MMA)</td>
<td>Plasma MMA</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>Plasma pyridoxal 5’ phosphate (PLP)</td>
<td>Plasma PLP (vitamin B&lt;sub&gt;6&lt;/sub&gt;)</td>
<td>Non-radioactive enzymatic assay</td>
</tr>
<tr>
<td>Plasma choline, betaine, DMG, TMAO</td>
<td>Plasma choline, betaine, DMG, TMAO</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>Plasma formate</td>
<td>Plasma formate</td>
<td>GC-MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Global DNA methylation and hydroxymethylation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38 SNPS in 26 one-carbon nutrient related genes</td>
</tr>
</tbody>
</table>
4.3 Baseline characteristics of the mothers

Baseline maternal characteristics are listed in Table 4.2. On average, the pregnant women were 32 years of age and had a BMI of approximately 25 at recruitment. Over half of the participants were Caucasian (56%) and 19% were of Asian ethnicity (including Chinese, Filipino, Japanese, Korean, and Southeast Asian). Other ethnicities included Black and Aboriginal and made up 19% of the cohort. Women were generally well educated with 83% reporting that they completed a college/vocational diploma (23%), university degree or more (60%). A small proportion of women reported household income below the poverty line (11%) and fewer women were single mothers (6%). Forty percent of mothers were experiencing their first pregnancy (gravidity = 1) and 54% were nulliparous (parity = 0).
### Table 4.2. Baseline maternal characteristics

<table>
<thead>
<tr>
<th>Descriptive characteristics</th>
<th>All subjects (n=364)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) [mean (95% CI)]</td>
<td>32 (31, 32)</td>
</tr>
<tr>
<td>Weeks gestation (wks) [mean (95% CI)]</td>
<td>13.1 (13.0, 13.3)</td>
</tr>
<tr>
<td>BMI at recruitment (kg/m^2) [mean (95% CI)]</td>
<td>24.6 (24.1, 25.1)</td>
</tr>
<tr>
<td>Ethnicity (%)^1</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>56</td>
</tr>
<tr>
<td>Asian</td>
<td>19</td>
</tr>
<tr>
<td>South Asian</td>
<td>6</td>
</tr>
<tr>
<td>Other</td>
<td>19</td>
</tr>
<tr>
<td>Born in Canada (%)^2</td>
<td>49</td>
</tr>
<tr>
<td>Education Level (highest degree/diploma attained) (%)^1</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>High School Diploma</td>
<td>15</td>
</tr>
<tr>
<td>College/Vocational Diploma</td>
<td>23</td>
</tr>
<tr>
<td>University degree or higher</td>
<td>60</td>
</tr>
<tr>
<td>Household income below poverty line (%)^2</td>
<td>11</td>
</tr>
<tr>
<td>Single parent family (%)^3</td>
<td>5.8</td>
</tr>
<tr>
<td>Gravidity (%)^4</td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td>60</td>
</tr>
<tr>
<td>Parity (%)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>54</td>
</tr>
<tr>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>&gt;1</td>
<td>14</td>
</tr>
<tr>
<td>Smoker (%)^5</td>
<td>7</td>
</tr>
</tbody>
</table>

^1 Missing 2 values, ^2 Missing 1 value, ^3 Missing 5 values, ^4 Missing 3 values, ^5 Missing 11 values
4.4 Maternal dietary and supplemental intakes of one-carbon nutrients

Maternal dietary intakes for folate, vitamins B₆ and B₁₂ and choline are listed in Table 4.3. Briefly, the prevalence of folate, vitamin B₁₂ and vitamin B₆ inadequacy (intakes of less than the EAR) from diet alone was approximately 45%, 11%, and 50%, respectively, at some point during pregnancy. Overall, dietary intakes did not change appreciably between early and late pregnancy. However, ubiquitous prenatal supplement use (> 90% during pregnancy) containing these vitamins at or above the RDA ensured adequate, if not excessive, intakes for these nutrients. However, prenatal vitamins do not contain choline, therefore over 85% of study participants had dietary intakes below the AI during pregnancy.

<table>
<thead>
<tr>
<th>Dietary Intake</th>
<th>Early Pregnancy [Mean (95% CI)]</th>
<th>Late pregnancy [Mean (95% CI)]</th>
<th>EAR for Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (µg DFE/d)</td>
<td>483 (461, 505)</td>
<td>465 (444, 486)</td>
<td>520</td>
</tr>
<tr>
<td>Vitamin B₆ (mg/d)</td>
<td>1.8 (1.7, 1.9)</td>
<td>1.7 (1.6, 1.8)</td>
<td>1.6</td>
</tr>
<tr>
<td>Vitamin B₁₂ (µg/d)</td>
<td>4.7 (4.4, 5.0)</td>
<td>4.6 (4.3, 4.9)</td>
<td>2.2</td>
</tr>
<tr>
<td>Choline (mg/d)</td>
<td>313 (298, 328)</td>
<td>305 (291, 319)</td>
<td>450 (AI)</td>
</tr>
</tbody>
</table>

DFE, dietary folate equivalent; EAR, estimated average requirement; AI, adequate intake.

Overall, 60.1% (95% CI: 55.8, 64.3) of the women reported using a B vitamin–containing supplement at least once each week 30 d before pregnancy. This increased to 92.8% (95% CI: 89.6, 95.2) in early pregnancy (between 0 and 16 wk gestation) and remained at 89.0% (95% CI: 85.0, 92.3) in late pregnancy (between 23 and 37 wk gestation). The most common type of B vitamin–containing supplement used before conception and in early and late pregnancy was a
prenatal multivitamin formulation, which contained folic acid, vitamin B\textsubscript{12}, vitamin B\textsubscript{6}, and other vitamins and minerals. Before conception, women consumed folic acid alone (13.7%), folic acid, vitamin B\textsubscript{12}, and vitamin B\textsubscript{6}–containing multivitamins designed for non-pregnant adults (11.1%), and vitamin B complex (7.6%), which contained folic acid, vitamin B\textsubscript{12}, and vitamin B\textsubscript{6}.

Furthermore, 19.7% (95% CI: 14.6, 25.7), 14.9% (95% CI: 11.2, 19.2), and 24.9% (95% CI: 19.8, 30.6) reported using a combination of ≥2 of the aforementioned B vitamin–containing supplements before and during early and late pregnancy, respectively.

Thirty days before conception and during early pregnancy, women reported median supplemental intakes of 1000 µg/d of folic acid, 1.9 mg/d of vitamin B\textsubscript{6}, and 2.6 mg/d of vitamin B\textsubscript{12}. The RDA for folate, vitamin B\textsubscript{6}, and vitamin B\textsubscript{12} during pregnancy is 600 µg of DFE/d, 1.9 mg/d, and 2.6 mg/d, respectively (1). None of the prenatal multivitamin formulations consumed contained choline or betaine.

4.5 Newborn infant characteristics

Newborn characteristics are described in Table 4.4. Seventy four percent of deliveries were vaginal births. On average, newborns were full term (>37 weeks gestation) and exhibited normal ranges for birth weight, length and head circumference. Apgar scores are a quick measure of the newborn’s tolerance of childbirth and how it is adjusting outside of the womb; a score above 7 is generally considered to be normal. Ninety five percent and 99% of newborns had an Apgar score above 7 at 1 minute and 5 minutes, respectively.
Table 4.4. Newborn characteristic

<table>
<thead>
<tr>
<th>Descriptive characteristics</th>
<th>All subjects (n=309)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at birth (wks)</td>
<td>39.3 (39.1, 39.5)</td>
</tr>
<tr>
<td>Caesarian delivery, n (%)</td>
<td>81 (26)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>173 (56)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3346 (3285, 3406)</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>51.9 (51.5, 52.2)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>34.2 (34.0, 34.5)</td>
</tr>
<tr>
<td>1 min. Apgar score above 7, n (%)</td>
<td>293 (95)</td>
</tr>
<tr>
<td>5 min Apgar score above 7, n (%)</td>
<td>305 (99)</td>
</tr>
</tbody>
</table>

Values expressed at mean (95% CI) unless otherwise specified.

4.6 Pregnancy and birth outcomes

There were two NTD-affected pregnancies in our cohort and one with a severe congenital heart defect, all which were terminated. Seventeen women (5%) were diagnosed with gestational diabetes, while 9 women (3%) developed pregnancy induced hypertension. There were 20 preterm births (<37 weeks gestation), with the earliest occurring at 28 weeks.

4.7 My contributions to the PREFORM study

I contributed to the study design and modified the protocol. I conducted the study and participated in all aspects of the study including recruitment of subjects, administration of health
questionnaire and FFQ, collection and processing of blood samples, sample analyses with appropriate collaboration, extraction of MNCs, DNA extraction, creation of a biorepository, and data analysis and interpretation. I coordinated collaborations with SickKids Hospital (UMFA analysis), Cornell University (choline analysis), A/C Diagnostics (PLP analysis), and Tufts University (DNA methylation and hydroxymethylation analysis).
Chapter 5: Concentrations of folate and unmetabolized folic acid in a cohort of pregnant Canadian women and umbilical cord blood.


The format has been modified from the original publication in order to conform to the format of this thesis.

5.1 Abstract

**Background:** Mandatory fortification, prevalent supplement use and public health guidelines recommending periconceptual supplementation have increased folic acid intakes among North American pregnant women. However, the effects of increased folic acid intakes during pregnancy on maternal and cord blood folate concentrations are not well established.

**Objectives:** This prospective study determined maternal and cord blood concentrations of folate and unmetabolized folic acid (UMFA) in a cohort of pregnant Canadian women and their newborns and examined the effect of maternal intake of folate and folic acid and fetal genetic variants in folate metabolism on folate status.

**Design:** Folate and folic acid intakes of 368 Canadian pregnant women were assessed in early (0-16 wks) and late (23-37 wks) pregnancy. Blood concentrations of folate and UMFA were
measured by immunoassays and LC-MS, respectively, in maternal samples in early pregnancy (12-16 wks) and at delivery (28-42 wks), and in cord blood. Four fetal genetic variants of the \textit{MTHFR} and \textit{DHFR} genes were assessed for their association with cord blood concentrations of folate and UMFA.

\textbf{Results:} Geometric mean (95\% CI) maternal red blood cell (RBC) folate concentrations were 2417 (2362, 2472) and 2793 (2721, 2867) nmol/L in early pregnancy and at delivery, respectively. Mean cord RBC folate concentration was 2689 (2614, 2765) nmol/L. UMFA was detectable in >90\% of maternal and cord plasma samples. While 3 fetal \textit{MTHFR} and \textit{DHFR} genetic variants had no effect, the fetal \textit{MTHFR} 677TT genotype was associated with significantly lower cord serum (p=0.03) and higher cord RBC (p=0.02) folate concentrations compared with the wild-type.

\textbf{Conclusions:} Notwithstanding differences in assays, maternal and cord RBC folate and plasma UMFA concentrations were higher than previously reported values. Functional ramifications of high folate and UMFA concentrations in maternal and fetal circulation warrant further investigation as excess folate status may impact long-term health outcomes of the offspring. This study is registered at www.clinicaltrials.gov (NCT#02244684).
5.2 Introduction

Folate mediates the transfer of one-carbon units involved in nucleotide biosynthesis and biological methylation reactions and hence, plays an important role in DNA synthesis, stability, repair, and methylation (395). Metabolic demand for folate is increased during pregnancy due to the rapid rate of maternal and fetal cellular growth and development (1). Inadequate folate status during pregnancy is associated with adverse pregnancy and birth outcomes including megaloblastic anemia and neural tube defects (1). In light of overwhelming evidence for a protective effect of periconceptional folic acid supplementation on the risk of neural tube defects, folic acid fortification of the food supply became mandatory in 1998 in the US and Canada (1, 4), leading to a 26-47% reduction in the rates of neural tube defects (3, 162). In addition, women of reproductive age are recommended to consume 400 µg folic acid daily from fortified foods or supplements, or both, 2-3 months prior to conception and during pregnancy (1). Studies from Canada and the US report the majority of women now consume supplements containing folic acid prior to and during pregnancy (189, 191). These facts suggest that North American pregnant women and their fetuses are likely exposed to high levels of folate and folic acid.

Longitudinal analyses of the folate status of Americans in the National Health and Nutrition Examination Survey (NHANES) report serum and red blood cell (RBC) folate concentrations are 2.5x and 1.5x pre-fortification values (101). In the Canadian Health Measures Survey, approximately 35% of Canadian women 20 to 39 years of age had RBC folate concentrations greater than the 97th percentile of NHANES (1999-2004) data (107). Finally, following folic acid fortification, most individuals have measurable concentrations of unmetabolized folic acid (UMFA) in blood, likely attributable to high synthetic folic acid intakes (49, 91, 92, 432).
Concerns have been raised over whether or not high synthetic folic acid intakes and circulating UMFA concentrations pose a health risk (5). These concerns include masking of vitamin B$_{12}$ deficiency (433), accelerated tumor progression (136, 140), aberrant DNA methylation (393), and diminished natural killer cell cytotoxicity (157). Furthermore, periconceptional high folic acid intake has been linked to aberrant embryonic developments in mice (32, 34) and humans (6); increased risk for asthma and respiratory tract infections (7) and insulin resistance and obesity in children (8); perturbation in DNA methylation programming (406); and increased cancer risk in the rodent offspring (79). Therefore, a more complete understanding of blood folate and UMFA concentrations in both the maternal and fetal compartments in a population with mandatory folic acid fortification and widespread folic acid supplement use is necessary. This will help to assess whether current folic acid fortification and supplementation policies provide sufficient coverage to yield maximal protection against neural tube defects without creating undue risk.

The present study assessed maternal and cord blood concentrations of folate and UMFA in a cohort of pregnant Canadian women and their newborn infants and determined the effect of maternal intake of dietary folate and supplemental folic acid on maternal and cord blood concentrations of folate and UMFA. As previous studies suggest that blood folate concentrations and specifically UMFA may be influenced by genetic variants in folate metabolism (173, 332), the effect of four variants in fetal methylenetetrahydrofolate reductase (MTHFR) and dihydrofolate reductase (DHFR) genes on cord blood concentrations of folate and UMFA was also examined.
5.3 Subjects and Methods

**Subjects and study design**

All procedures and protocols were reviewed and approved by St. Michael’s Hospital Research Ethics Board, and written informed consent was obtained from all women during the initial study visit. Between November 2010 and January 2012, 368 healthy women between the ages of 18 and 45 years with an uncomplicated singleton pregnancy between 12 and 16 wk of gestation were recruited to participate in the PREFORM (PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant) study (ClinicalTrials.gov identifier: NCT02244684). The aim of the PREFORM study is to determine the effects of maternal intake of folate and folic acid on DNA methylation of mononuclear cells (MNCs) in cord blood. The study design and subject characteristics have been reported in detail previously (Chapter 3 and ref. (434)). Briefly, women attending a prenatal clinic visit were recruited from obstetrician offices at St. Michael’s Hospital, situated in the downtown core of Toronto, Canada. St. Michael’s Hospital serves a demographically diverse inner city population and delivers over 3000 neonates annually. At enrolment, the participants completed a Baseline Demographic and Health Questionnaire based on the Canadian Community Health Survey, Cycle 2.2 (435) to collect information regarding demographics and health status as described previously (Chapter 3 and ref. (434)). Women in the study delivered between April 2011 and August 2012. At birth, the infants’ anthropometric information was recorded.

**Assessment of dietary and supplemental intake**

Maternal dietary intakes of folate and other one-carbon nutrients during early and late pregnancy
were captured using a 110-item semi-quantitative Block food frequency questionnaire (FFQ; NutritionQuest, Berkeley, CA) administered during the first (12-16 wks gestation) and second (35-37 wks gestation) study visits as described previously (Chapter 3 and ref. (434)). Briefly, the Block FFQ is designed to capture the consumption of specific foods and beverages in the previous three months and specifically between 0 and 16 wks (early pregnancy) and between 23 and 37 wks of gestation (mid to late pregnancy) in the present study. The Block FFQ has been validated for B vitamin intake in a sample of Ontarian women of reproductive age (34-36 yrs) (430). The median deattenuated Pearson correlation coefficient between the Block FFQ and two 24-h recalls was reported to be 0.76 for folate (430). Vitamin supplement use was assessed for three time-periods: pre-conception (30 d prior to pregnancy), early pregnancy (conception to 16 wks gestation) and mid to late pregnancy (23 to 37 wks gestation) using the Baseline Demographic Questionnaire and the Block FFQ. Information on the type, frequency and dose of folic acid supplement was obtained for the preconception and early pregnancy time points. In mid to late pregnancy, information on the type and frequency, but not the dose, of folic acid supplement use was collected.

**Sample collection and processing**

Maternal venous blood was drawn from each subject during early pregnancy (12-16 wks gestation) and at delivery (28-42 wks gestation). The participants were not instructed to fast prior to blood collection and the time of last meal and/or supplement use was recorded. The average (± SD) length of time food and/or supplements were consumed prior to blood draw was 5.2 ± 2.4 hrs. At delivery, up to 20 mL of venous blood was also obtained from the umbilical cord. Blood samples were collected in evacuated tubes containing EDTA for plasma homocysteine and RBC folate analysis or containing clot activator and gel for serum separation for determination of
serum folate. Plasma and serum were separated from whole blood within two hours of collection and serum and RBC folate and plasma homocysteine were determined immediately in the St. Michael’s Hospital Core Laboratory. The remaining plasma was stored at -80°C until further analysis for plasma UMFA. Approximately 8 mL of cord blood was used for MNCs extraction using the ACCUSPIN System-Histopaque 1077 System (Sigma-Aldrich, St. Louis, MO, USA). Two hundred µL RNAProtect Cell Reagent (Qiagen Sciences, Germantown, MD, USA) was added to cell pellets before freezing. Cell pellets were stored at -80°C until DNA extraction.

**Serum and RBC folate assays**

Serum and RBC folate concentrations were determined in the St. Michael’s Hospital Core Laboratory using quantitative, high throughput protein-binding immunoassays, the Access Folate Assay (Beckman Coulter, Brea, CA, USA) and the Elecsys Folate Assay (Roche Diagnostics, Mannheim, Germany), respectively, which utilize folate-binding protein with chemiluminescence detection. Intra- and Inter-assay coefficients of variation (CVs) were <5% for the Access Folate Assay (serum) and <10% and <15%, respectively, for the Elecsys Folate System (RBC). The accuracy and precision of serum folate concentrations assessed by the Access Folate Assay were determined using National Institute of Standards and Technology Standard SRM 1955 Homocysteine and Folate in Frozen Human Serum. The mean serum folate concentrations (± SD; n=3) determined by the Access Folate System were 4.8 ± 0.1, 10.5 ± 0.4, and 29.1 ± 2.0 nmol/L at the low, medium and high SRM 1955 certified to contain 4.5, 10, and 25 nmol/L, respectively. The CV for each SRM 1955 level was 1.5%, 3.4%, and 6.8%, respectively. We also compared RBC folate concentrations measured by the Elecsys Folate System with those determined by microbiologic assay using chloramphenicol-resistant *Lactobacillus rhamnosus* with 5-methylTHF as the calibrator (98) in a convenience sample.
(n=16) of healthy male and non-pregnant female volunteers (age range: 20-63 yrs). The RBC folate values obtained with the Elecsys Folate Assay were positively correlated with those using the microbiologic assay (Pearson r=0.90, p<0.0001). On average, RBC folate concentration values were 5.3% (40 nmol/L) higher with the Elecsys Folate Assay compared with the microbiologic assay (1171 ± 184 nmol/L versus 1131 ± 247 nmol/L) with all values within ± 20%.

**Plasma UMFA assay**

Standards and sample preparation for determination of plasma UMFA was conducted in the co-authors (SA, DLO) laboratory and analyzed at the Analytical Facility for Bioactive Molecules at The Hospital for Sick Children, Toronto, Canada using the stable isotope dilution liquid chromatography tandem mass spectrometry according to methods published by Pfeiffer et al (436). Standard curves were prepared from spectrometrically verified stock solutions of folic acid and folic acid-13C5 (Merck Eprova, Switzerland). Based on a signal:noise ratio of >3:1, the limit of detection was 0.2 nmol/L. Using duplicate sample preparations from 71 subjects, the intra-assay CV for plasma UMFA for concentrations <0.9 nmol/L, 0.9-2.0 nmol/L and >2.0 nmol/L was 12.3%, 16.8%, and 4%, respectively. Inter-assay accuracy and precision was also determined using Levels II (certified value = 1.05 ± 0.16 nmol/L) and III (1.07 ± 0.24 nmol/L) of SRM 1955 (n=5 runs). The mean ± SD (CV) of the Level II and Level III reference material was 1.24 ± 0.12 nmol/L (9.5%) and 1.19 ± 0.12 nmol/L (10.0%), respectively.

**Plasma homocysteine assay**

Total plasma homocysteine (an inverse indicator of folate status (1)) concentrations were determined by a quantitative, enzymatic assay using the Synchron LX20 System (Beckman...
Coulter, Brea, CA) in the St. Michael’s Hospital Core Laboratory. Intra- and inter-assay CVs were < 5.5% and <2%, respectively.

**DNA isolation and genotyping**

DNA from cord blood MNCs was isolated using the DNA Isolation Kit for Mammalian Blood (Roche Diagnostics, Mannheim, Germany). The extracted DNA was > 20 kb in all instances, was free of RNA contamination, and had an A$_{260/280}$ ratio between 1.8 and 2.0.

We examined the effects of two single nucleotide polymorphisms in the gene encoding MTHFR, which catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF (5), and two variants in the gene encoding DHFR, which converts folic acid and dihydrofolate to tetrahydrofolate (THF) (5), on cord blood folate and homocysteine concentrations. The MTHFR $677C>T$ (rs1801133) and $1298A>C$ (rs1801131) polymorphisms were genotyped using TaqMan genotyping assays on an ABI Vii7 Biosystem (Applied Biosystems, Foster City, CA, USA) as described previously (437). The DHFR rs1643649 located in intron 3 was genotyped using the iPLEX Assay with the MassARRAY platform (Sequenom, San Diego, CA, USA) at the Clinical Genomics Centre (Toronto, Canada) as described previously (438). The DHFR 19bp insertion/deletion (rs70991108) was assessed by PCR with fluorescent hybridization as previously described (332).

**Statistical analysis**

Maternal and cord serum and RBC folate and plasma UMFA concentrations were log transformed to meet the assumptions of normality. The smallest detectable concentration (e.g., limit of detection) of plasma UMFA was 0.2 nmol/L. For the analyses, plasma UMFA
concentration values < 0.2 were considered undetectable and were assigned a value of 0. Descriptive statistics were reported as mean and geometric mean for normally distributed and transformed variables, respectively, with corresponding 95% confidence intervals (CI). Categorical variables were reported as n (%). T-tests and chi-square tests were used to determine differences between supplement users and nonusers with respect to continuous and categorical variables, respectively. Correlations between dietary and blood parameters were assessed using Pearson’s tests. Least squares regression and generalized least squares regression was used to identify predictors of cord and maternal concentrations. Potential predictors from the literature for maternal blood concentrations included maternal age, BMI, education level, ethnicity, dietary folate equivalents and supplemental folic acid intake in early pregnancy, and fasting time before blood draw (107, 171). Potential predictors for cord blood concentrations included maternal dietary and supplemental folate intake in late pregnancy, maternal and cord folate concentrations, parity and infant MTHFR677C>T genotype. Differences in cord blood parameters among fetal genotypes were adjusted for ethnicity (white/nonwhite), gestational age at delivery, and dietary folate and supplemental folic acid intake in late pregnancy. Logistic regression was used to determine differences in percent of detectable UMFA (≥ 0.2 nmol/L) among fetal genotypes.

All tests were two-tailed and an alpha level < 0.05 was considered statistically significant. P-values for tests of interactions between fetal genotype and quartile of maternal RBC folate were adjusted using the false discovery rate adjustment using PROC MULTTEST in order to maintain an overall significance level of 0.05. Statistical analyses were performed using SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA). All available data was included in analyses and the samples size for each analysis was reported.
5.4 Results

Characteristics of the study subjects and newborn infants

Detailed information on the PREFORM study design and participants has been published previously (Chapter 3 and ref. (434)). The majority of subjects were Caucasian, university graduates, nulliparous, nonsmokers, and the mean (± SD) age was 32 ± 5 years old (Table 5.1). Maternal characteristics were not significantly different between folic acid-containing supplement users and nonusers. Newborn gestational age, birth weight, length and head circumference were within normal ranges (Table 5.2). There were no differences in gestational age at birth, sex of infant, birth weight, birth length or head circumference between newborns from mothers who used supplements throughout pregnancy and those from mothers who did not.

Table 5.1. Maternal characteristics by supplement use

<table>
<thead>
<tr>
<th>Descriptive characteristics</th>
<th>All subjects</th>
<th>Early pregnancy folic acid supplement users</th>
<th>Early pregnancy folic acid supplement nonusers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants, n</td>
<td>364</td>
<td>339</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>32 (31, 32)</td>
<td>32 (31, 32)</td>
<td>31 (29, 34)</td>
<td>0.37</td>
</tr>
<tr>
<td>Weeks gestation (wks)</td>
<td>13.1 (13.0, 13.3)</td>
<td>13.2 (12.9, 13.4)</td>
<td>13.1 (12.3, 14.0)</td>
<td>0.96</td>
</tr>
<tr>
<td>BMI at recruitment (kg/m²)</td>
<td>24.6 (24.1, 25.1)</td>
<td>24.4 (23.9, 24.9)</td>
<td>26.6 (24.1, 29.1)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Ethnicity (%)

<table>
<thead>
<tr>
<th>Participants, n</th>
<th>362</th>
<th>337</th>
<th>25</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>203 (56)</td>
<td>189 (56)</td>
<td>14 (54)</td>
<td>0.71</td>
</tr>
<tr>
<td>Asian</td>
<td>70 (19)</td>
<td>67 (20)</td>
<td>3 (15)</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>21 (6)</td>
<td>20 (6)</td>
<td>1 (4)</td>
<td></td>
</tr>
<tr>
<td>Other²</td>
<td>68 (19)</td>
<td>61 (18)</td>
<td>7 (27)</td>
<td></td>
</tr>
<tr>
<td>Descriptive characteristics</td>
<td>All subjects</td>
<td>Early pregnancy folic acid supplement users</td>
<td>Early pregnancy folic acid supplement nonusers</td>
<td>p-value</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------------</td>
<td>-------------------------------------------</td>
<td>---------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Early pregnancy folic acid supplement users</td>
<td>Early pregnancy folic acid supplement nonusers</td>
<td></td>
</tr>
<tr>
<td>Education Level (highest degree/diploma attained) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Participants, n</td>
<td>363</td>
<td>338</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>7 (2)</td>
<td>6 (2)</td>
<td>1 (4)</td>
<td>0.63</td>
</tr>
<tr>
<td>High School Diploma</td>
<td>55 (15)</td>
<td>50 (15)</td>
<td>5 (23)</td>
<td></td>
</tr>
<tr>
<td>College/Vocational Diploma</td>
<td>83 (23)</td>
<td>78 (23)</td>
<td>5 (19)</td>
<td></td>
</tr>
<tr>
<td>University degree or higher</td>
<td>218 (60)</td>
<td>204 (60)</td>
<td>14 (54)</td>
<td></td>
</tr>
<tr>
<td>Gravidity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Participants, n</td>
<td>361</td>
<td>336</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>144 (40)</td>
<td>132 (39)</td>
<td>12 (46)</td>
<td>0.46</td>
</tr>
<tr>
<td>&gt;1</td>
<td>217 (60)</td>
<td>204 (61)</td>
<td>13 (54)</td>
<td></td>
</tr>
<tr>
<td>Parity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Participants, n</td>
<td>362</td>
<td>337</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>193 (54)</td>
<td>180 (53)</td>
<td>13 (50)</td>
<td>0.92</td>
</tr>
<tr>
<td>1</td>
<td>119 (32)</td>
<td>110 (33)</td>
<td>9 (35)</td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td>50 (14)</td>
<td>47 (14)</td>
<td>3 (15)</td>
<td></td>
</tr>
<tr>
<td>Smoker (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Participants, n</td>
<td>353</td>
<td>328</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>23 (7)</td>
<td>20 (6)</td>
<td>3 (1)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Skewed distributions log transformed prior to analysis. Mean (95% CI) or geometric mean (back-transformed 95% CI) reported for continuous variables; n (%) reported for categorical variables. T-tests used to compare continuous variables and chi-square tests used to compare categorical variables, except for Fisher’s exact test for maternal smoking status. P-value testing the null hypothesis of no difference between supplement users and nonusers. 183% reported consuming at least 1mg folic acid/d in 0-16 wks gestation. 2Ethnic group ‘Other’ includes Black and Aboriginal. Maternal sample size range: 353-364 due to missing values. *folic acid-containing supplement users in 0 - 16 wks gestation.
Table 5.2. Newborn characteristics by supplement use

Mean (95% CI) reported for continuous variables; n (%) reported for categorical variables. T-tests used to compare continuous variables and chi-square tests used to compare categorical variables. P-value testing the null hypothesis of no difference between supplement users and nonusers.

Dietary and supplemental intakes of folate and folic acid

Intakes and blood concentrations of folate and folic acid in the PREFORM Study are summarized in Figure 5.1. Detailed information on dietary and supplemental intakes of folate and folic acid of the PREFORM study participants has been discussed previously (Chapter 3 and ref. (434)). Briefly, mean (± SD) maternal dietary folate (naturally occurring folate and folic acid added as a fortificant) intakes were 483 ± 203 µg DFE/d in early pregnancy and 465 ± 186 µg
DFE/d in late pregnancy. Ninety-three percent and 90% of women reported using folic acid-containing supplements in early and mid to late pregnancy, respectively; 83% reported daily doses of at least 1000 µg folic acid in early pregnancy (434). The median (interquartile range, IQR) maternal supplemental folic acid intake was 1000 (1000, 1000) µg/d during early pregnancy. Self-reported supplemental vitamin use in mid to late pregnancy was only reported as a categorical variable (yes/no) and therefore, no supplemental folic acid intake dosages were reported for mid to late pregnancy.

Figure 5.1. Schematic of folate and folic acid intakes and blood concentrations in the PREFORM Study. DFE, dietary folate equivalent.
Maternal and cord blood folate and homocysteine concentrations

All subjects had serum and RBC folate concentrations above established deficiency cutoffs (<7 nmol/L and <305 nmol/L, respectively (434)) (Table 5.3). Geometric mean (95% CI) serum and RBC folate concentrations were 51 (49, 54) and 2417 (2362, 2472) nmol/L and 39 (37, 41) and 2793 (2721, 2867) nmol/L in early pregnancy (12-16 wks) and at delivery (38-42 wks), respectively. Geometric mean (95% CI) cord serum and RBC folate concentrations were 64 (61, 68) and 2689 (2614, 2765) nmol/L. No woman in early pregnancy or at delivery or cord sample had a plasma total homocysteine concentration >13 µmol/L.
### Table 5.3. Maternal and cord blood folate concentrations

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Folic acid supplement users*</th>
<th>Folic acid supplement nonusers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal concentrations at 12-16 wks gestation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=345</td>
<td></td>
<td>n=321</td>
<td>n=24</td>
<td></td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>51 (49, 54)</td>
<td>52 (49, 55)</td>
<td>47 (33, 69)</td>
<td>0.57</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>2417 (2362, 2472)</td>
<td>2423 (2367, 2480)</td>
<td>2343 (2086, 2632)</td>
<td>0.49</td>
</tr>
<tr>
<td>Plasma UMFA, nmol/L</td>
<td>2.41 (1.99, 2.88)</td>
<td>2.44 (2.01, 2.92)</td>
<td>2.37 (0.69, 5.71)</td>
<td>0.94</td>
</tr>
<tr>
<td>Plasma homocysteine, µmol/L</td>
<td>5.0 (4.9, 5.2)</td>
<td>5.0 (4.9, 5.2)</td>
<td>5.3 (4.6, 5.9)</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Maternal concentrations at delivery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=293</td>
<td></td>
<td>n=264</td>
<td>n=29</td>
<td></td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>39 (37, 41)</td>
<td>41 (39, 44)</td>
<td>23 (19, 28)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>2793 (2721, 2867)</td>
<td>2862 (2790, 2935)</td>
<td>2262 (2039, 2509)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma homocysteine, µmol/L</td>
<td>6.0 (5.8, 6.2)</td>
<td>6.0 (5.8, 6.2)</td>
<td>6.3 (5.7, 7.0)</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Cord blood concentrations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=285</td>
<td></td>
<td>n=257</td>
<td>n=28</td>
<td></td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>64 (61, 68)</td>
<td>67 (64, 70)</td>
<td>45 (38, 52)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>2689 (2614, 2765)</td>
<td>2741 (2663, 2822)</td>
<td>2337 (2136, 2555)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Plasma UMFA, nmol/L</td>
<td>0.68 (0.60, 0.77)</td>
<td>0.72 (0.62, 0.82)</td>
<td>0.47 (0.28, 0.68)</td>
<td>0.07</td>
</tr>
<tr>
<td>Plasma homocysteine, µmol/L</td>
<td>4.8 (4.7, 5.0)</td>
<td>4.7 (4.6, 4.9)</td>
<td>5.3 (4.8, 5.9)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Skewed distributions transformed prior to analysis. Mean (95% CI) or geometric mean (back-transformed 95% CI) reported for continuous variables. T-tests were used to compare continuous variables. P-value testing the null hypothesis of no difference between supplement users and nonusers. For total subjects, values with different superscript letters within each folate biomarker are significantly different from corresponding values at different time points: maternal blood values at delivery significantly different from those at 12-16 wks gestation (p<0.0001); cord blood values significantly different from corresponding maternal blood values at delivery (p<0.02); and cord blood values significantly different from corresponding maternal blood values at 12-16 wks gestation (p<0.0001). Maternal blood at 12-16 wks gestation sample size range: 342-345 due to missing values. Maternal blood at delivery sample size range: 285-293 due to missing values. Cord blood sample size range: 246-285 due to missing values. *For maternal concentrations at 12-16 weeks gestation, supplement use was during early pregnancy and for maternal concentrations at delivery and cord blood concentrations, supplement use was during late pregnancy. Participant recruitment occurred at 12-16 wks gestation when maternal blood was collected. UMFA, unmetabolized folic acid.
Maternal serum folate concentrations significantly decreased, while RBC folate and plasma homocysteine concentrations significantly increased, during pregnancy (p<0.0001). Cord serum folate concentrations were 64% higher than maternal serum folate concentrations at delivery (p<0.0001). In contrast, cord RBC folate concentrations were 3.8% lower than maternal RBC folate concentrations at delivery (p=0.01). Cord plasma homocysteine concentrations were 20% lower than maternal plasma homocysteine concentrations at delivery (p<0.0001). Relationships between maternal and cord blood remained the same when the statistical analyses were performed on those subjects who had a complete set of blood samples at each time point (i.e., early pregnancy, delivery, cord blood).

Serum or RBC folate or plasma homocysteine concentrations during early pregnancy were not significantly different between those who took folic acid-containing supplements and those who did not. In contrast, women who supplemented in late pregnancy had significantly higher serum (p<0.0001) and RBC folate (p<0.0001) concentrations at delivery than nonusers, while plasma homocysteine concentrations were not significantly different between the two groups.

Serum (p<0.0001) and RBC folate (p=0.0002) concentrations were also significantly higher in cord blood from mothers who supplemented in late pregnancy than in cord blood from nonusers. Plasma homocysteine concentrations were significantly lower in cord blood from mothers who took supplements in late pregnancy than in cord blood from nonusers (p=0.03).

**Maternal and cord blood UMFA concentrations**

During early pregnancy, maternal plasma UMFA was detectable (≥ 0.2 nmol/L) in 97% of the women (range: undetectable to 244 nmol/L) (Table 5.3). In maternal samples collected during
early pregnancy with detectable UMFA, the median (IQR) proportion of plasma UMFA to total serum folate was 3.09 (1.90, 8.93) %. Plasma UMFA was detectable in 93% of cord blood samples (range: undetectable to 15 nmol/L). The median (IQR) proportion of plasma UMFA to total serum folate in detectable cord blood samples was 1.34 (0.92, 2.12) %. Cord plasma UMFA concentrations were 72% lower than maternal plasma UMFA concentrations during early pregnancy (p<0.0001). The proportion of plasma UMFA making up total serum folate was greater for maternal than cord blood (p<0.0001).

Maternal plasma UMFA concentrations during early pregnancy were not significantly different between those who took folic acid-containing supplements and those who did not. Plasma UMFA concentrations were not significantly higher in cord blood from mothers who supplemented in late pregnancy than in cord blood from nonusers (p=0.07).

RBC folate and plasma UMFA concentrations were weakly correlated in maternal blood during early pregnancy (r=0.18, p=0.0009) but not in cord blood (r=0.08, p=0.23). In contrast, serum folate and plasma UMFA concentrations were strongly correlated in maternal blood during early pregnancy (r=0.81, p<0.0001) and were weakly correlated in cord blood (r=0.24, p=0.0002).

**Association of maternal dietary and supplemental intakes with blood concentrations**

Previously reported maternal dietary intakes of folate (naturally occurring and folic acid added as a fortificant) (434) were not associated with maternal folate concentrations either in early pregnancy or at delivery (data not shown). In unadjusted general linear models, folic acid supplement use in early pregnancy predicted maternal serum and RBC folate (both p=0.03), but not plasma UMFA, concentrations in early pregnancy (**Table 5.4**). However, only serum folate concentrations remained significant after adjusting for covariates (p=0.0007). Maternal dietary
intakes of folate in late pregnancy were not associated with cord folate concentrations (data not shown).

Table 5.4. Regression analyses of the association of maternal supplemental folic acid intake (mg/d) in early pregnancy with maternal concentrations of serum and RBC folate, plasma UMFA and plasma homocysteine at recruitment (12-16 wks gestation)

<table>
<thead>
<tr>
<th>Maternal blood parameter</th>
<th>Unadjusted model</th>
<th>Adjusted model*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R^2 )</td>
<td>( \beta )</td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Plasma UMFA, nmol/L</td>
<td>0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma homocysteine, µmol/L</td>
<td>0.00</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

Data analyzed using general linear models. Adjusted model designed using stepwise regression analysis with entry and stay cutoffs at 0.15. *Models were additionally adjusted for time of last meal before blood draw, maternal age, BMI, education level and ethnicity, parity, and maternal dietary total folate and folic acid intake in early pregnancy. Regression analysis was performed with maternal supplemental folic acid intake in early pregnancy as independent variables and maternal concentrations of serum and RBC folate, UMFA and homocysteine as dependent variables. Maternal serum and RBC folate and plasma UMFA concentrations were log transformed prior to analysis. Sample size range: 283-322 due to missing values. UMFA, unmetabolized folic acid.

Association between maternal and cord blood concentrations

In unadjusted general linear models, maternal concentrations of serum and RBC folate and plasma homocysteine at delivery were positively associated with concentrations of the
corresponding biomarker in the cord blood (all p<0.0001) (Table 5.5). Adjusting for additional covariates did not change the associations; in all three analyses, the adjusted estimates were similar to the unadjusted estimates.

Table 5.5. Regression analyses of the association of maternal concentrations of serum and RBC folate and plasma homocysteine at delivery with the corresponding cord concentrations

<table>
<thead>
<tr>
<th>Cord blood parameter</th>
<th>Unadjusted model</th>
<th>Adjusted model*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate, nmol/L</td>
<td>R² 0.14 β 1.73 95% CI (1.19, 2.28) p &lt;0.0001</td>
<td>R² 0.31 β 3.39 95% CI (0.95, 1.94) p &lt;0.0001</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>R² 0.23 β 0.08 95% CI (0.06, 0.09) p &lt;0.0001</td>
<td>R² 0.30 β 0.08 95% CI (0.06, 0.09) p &lt;0.0001</td>
</tr>
<tr>
<td>Plasma homocysteine, µmol/L</td>
<td>R² 0.42 β 0.57 95% CI (0.50, 0.65) p &lt;0.0001</td>
<td>R² 0.50 β 0.57 95% CI (0.47, 0.63) p &lt;0.0001</td>
</tr>
</tbody>
</table>

Data analyzed using general linear models. Adjusted model designed using stepwise regression analysis with entry and stay cutoffs at 0.15. *Models were additionally adjusted for other maternal and cord folate parameters, parity and infant MTHFR677C>T genotype. Regression analysis was performed with maternal concentrations of serum and RBC folate and plasma homocysteine at delivery as independent variables and cord concentrations of serum and RBC folate and plasma homocysteine as dependent variables. Cord serum and RBC folate concentrations were log transformed prior to analysis. Sample size range: 223-275 due to missing values.

Cord blood folate and homocysteine concentrations based on infant genotype

All genotypes were found to be in Hardy-Weinberg equilibrium (p>0.22), indicating the observed genotype frequencies were not different from the expected frequencies derived from the Hardy-Weinberg equation. Minor allele frequency for all genotypes was >5%
(MTHFR677C>T rs1801133, 29.9%; MTHFR1298A>C rs1801131, 30.5%; DHFR intron 3 SNP rs1643649 22.5%; DHFR 19bp insertion/deletion rs70991108, 46.2%). The MTHFR 1298A>C or DHFR 19-bp insertion/deletion genotype in cord blood MNCs was not significantly associated with serum and RBC folate, plasma UMFA, or plasma homocysteine concentrations or proportion of detectable plasma UMFA in cord blood (Table 5.6). However, with the MTHFR 677C>T variant, cord serum folate concentrations were higher in the wild-type CC newborns than in the homozygous TT newborns (p=0.03) (Table 5.7). In contrast, higher RBC folate concentrations were found in cord blood in the homozygous TT newborns compared with the wild-type CC newborns (p=0.02). With respect to the DHFR intron 3 polymorphism, cord plasma homocysteine concentrations differed across the genotypes (p=0.01). Cord plasma homocysteine concentrations were lower in the homozygous TT newborns than in the heterozygous CT newborns (p=0.036), while there was no difference between the wild-type CC and heterozygous CT variant. The effect of interactions between infant genotype and quartiles of maternal RBC folate concentrations at delivery on serum and RBC folate, plasma UMFA, and plasma homocysteine concentrations in cord blood were determined. However, after adjusting for multiple testing using the false discovery rate in PROC MULTTEST, no significant interactions were found (data not shown).
Table 5.6. Folate, folic acid and homocysteine concentrations in umbilical cord blood according to fetal genotype

<table>
<thead>
<tr>
<th></th>
<th>MTHFR 1298A&gt;C</th>
<th></th>
<th>DHFR 19bp insertion/deletion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1801131</td>
<td></td>
<td></td>
<td>rs70991108</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>AC</td>
<td>CC</td>
<td>Ins/ins</td>
</tr>
<tr>
<td></td>
<td>(50%)</td>
<td>(39%)</td>
<td>(11%)</td>
<td>(29%)</td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>53 (48, 59)</td>
<td>57 (51, 64)</td>
<td>52 (43, 61)</td>
<td>55 (49, 62)</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>2480 (2354, 2613)</td>
<td>2555 (2419, 2699)</td>
<td>2583 (2365, 2821)</td>
<td>2581 (2430, 2741) (2401, 2670) (2260, 2593)</td>
</tr>
<tr>
<td>Plasma UMFA, nmol/L</td>
<td>0.64 (0.50, 0.82)</td>
<td>0.53 (0.38, 0.69)</td>
<td>0.55 (0.31, 0.84)</td>
<td>0.61 (0.44, 0.79) (0.45, 0.79) (0.34, 0.75)</td>
</tr>
<tr>
<td>Detectable UMFA, %</td>
<td>110 (49)</td>
<td>90 (40)</td>
<td>26 (11)</td>
<td>67 (30)</td>
</tr>
<tr>
<td>Plasma Hcy, µmol/L</td>
<td>4.9 (4.6, 5.2)</td>
<td>5.1 (4.8, 5.5)</td>
<td>5.1 (4.6, 5.7)</td>
<td>5.0 (4.6, 5.4)</td>
</tr>
</tbody>
</table>

Serum and RBC folate and UMFA were log transformed prior to analysis. Folate and folic acid concentrations reported as geometric mean (95% CI) and homocysteine concentrations reported as mean (95% CI). Detectable UMFA (limit of detection ≥ 0.2 nmol/L) reported as n (%). Folate, folic acid, and homocysteine concentrations compared using ANCOVA with Tukey’s post hoc adjustment. UMFA (limit of detection ≥ 0.2 nmol/L) was compared using logistic regression. All models adjusted for ethnicity, gestational age at delivery, and dietary folate and supplemental folic acid intake in late pregnancy. Sample size range 238-285 due to missing values. Hcy, homocysteine; Ins, allele without 19-bp deletion; del, allele with 19-bp deletion; UMFA, unmetabolized folic acid.
Table 5.7. Folate, folic acid and homocysteine concentrations in umbilical cord blood according to fetal genotype

<table>
<thead>
<tr>
<th></th>
<th>MTHFR 677C&gt;T rs1801133</th>
<th>DHFR intron 3 rs1643649</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC (51%)</td>
<td>CT (40%)</td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>57 (51, 63)</td>
<td>55 (49, 61)</td>
</tr>
<tr>
<td>RBC folate, nmol/L</td>
<td>2477 (2351, 2610)</td>
<td>2520 (2387, 2660)</td>
</tr>
<tr>
<td>Plasma UMFA, nmol/L</td>
<td>0.64 (0.48, 0.81)</td>
<td>0.57 (0.42, 0.74)</td>
</tr>
<tr>
<td>Detectable UMFA, %</td>
<td>120 (53)</td>
<td>87 (39)</td>
</tr>
<tr>
<td>Plasma Hcy, µmol/L</td>
<td>4.9 (4.6, 5.3)</td>
<td>5.1 (4.8, 5.5)</td>
</tr>
</tbody>
</table>

Serum and RBC folate and UMFA were log transformed prior to analysis. Folate and folic acid concentrations reported as geometric mean (95% CI) and homocysteine concentrations reported as mean (95% CI). Detectable UMFA (limit of detection ≥0.2 nmol/L) reported as n (%). Folate, folic acid, and homocysteine concentrations compared using ANCOVA with Tukey’s post hoc adjustment. UMFA (limit of detection ≥0.2 nmol/L) was compared using logistic regression. All models adjusted for ethnicity, gestational age at delivery, and dietary folate and supplemental folic acid intake in late pregnancy. Sample size range 238-285 due to missing values. Hcy, homocysteine; UMFA, unmetabolized folic acid.
5.5 Discussion

In a cohort of demographically diverse pregnant women from Southern Ontario, Canada, >90% of whom were taking a periconceptional folic acid-containing supplement, with the most common dose of 1000 µg/d, no subject was folate deficient. Most importantly, all women had RBC folate concentrations above 1000 nmol/L in early pregnancy, the estimated level below which the risk of neural tube defects begins to rise (434). Serum and RBC folate concentrations in early pregnancy and at delivery were in the range generally considered to be high. Different assays employed across the published studies and limitations associated with these assays preclude direct comparison of our data with others (169). Nevertheless, RBC folate concentrations measured by the Roche Elecsys immunoassay in our subjects are well above those previously reported (96-99, 439). For example, in the Canadian Health Measures Survey, the 50th percentile (95% CI) of RBC folate measured by the Siemens Immulite 2000 immunoassay for women 15-45 y was 1193 (1104, 1282) nmol/L with the 97th percentile value of 2299 (2021, 2577) nmol/L (50, 93, 103, 172, 173, 191, 194, 195). Using 1999-2006 NHANES data (107), it was recently reported median (95% CI) RBC folate measured by the Bio-Rad Quantaphase II assay and adjusted for the microbiologic assay for folic acid users and nonusers were 1628 (1589, 1695) and 1041 (962, 1184) nmol/L, respectively. The observed higher RBC folate concentrations in the present study are likely related to a higher proportion of women consuming folic acid supplements during the first trimester (93% versus ~55%), a higher dose of folic acid (mean daily dose of 1000 µg versus median daily dose of 800 µg) in the present study compared with the NHANES study, and the differences in analytic methods as will be discussed later.
Maternal and cord RBC folate concentrations in our study were much higher, whereas maternal and cord plasma homocysteine concentrations were generally comparable to or lower, than previously reported in countries without mandatory or with voluntary folic acid fortification only (191). These differences are likely due to mandatory folic acid fortification and ubiquitous folic acid supplement use in our cohort, as well as analytical differences in folate measurement (50, 89, 93, 172, 173, 193, 194, 414, 416).

Consistent with previous studies (96, 98), we observed higher serum folate and lower plasma homocysteine concentrations in cord blood than in maternal blood at delivery. In contrast to previous studies (50, 172, 173), cord RBC folate concentrations were 3.8% lower than maternal RBC folate concentrations at delivery. Serum and RBC folate concentrations were significantly higher, while plasma homocysteine concentrations were significantly lower, in cord blood from mothers on folic acid supplements in late pregnancy than in cord blood from nonusers.

Plasma UMFA was detectable in >90% of maternal and umbilical cord blood samples. Folic acid intake as little as 280 µg saturates the capacity of DHFR, a critical step in folic acid metabolism (173, 440), and results in its appearance unaltered in circulation (441). Plasma UMFA has been previously detected in pregnant women and umbilical cord from countries with lower periconceptional folic acid supplement use or without mandatory folic acid fortification (49, 93). Therefore, it is not surprising maternal and cord plasma UMFA concentrations in the present study were higher than those previously reported (50, 93). Consistent with a previous study (50, 93), the lower concentration and percentage of plasma UMFA contributing to total cord blood folate, and a weak or no correlation between plasma UMFA and serum and RBC folate in cord blood suggest that UMFA does not accumulate in the fetus, even with high folate status and detectable UMFA in mothers. The observed lower cord plasma UMFA versus maternal plasma
UMFA concentrations could be due to physiological limits in the transport of folic acid across the placenta. All three folate receptors and transporters are expressed in the term placenta (442). The lack of accumulation of UMFA in cord blood could be due to fundamental differences in folate and folic acid transport in the placenta compared with the small intestine. The other explanation is that UMFA is utilized quickly once in the placenta/fetal compartment and therefore does not accumulate in the inactive folate form (443). Therefore, the UMFA reaching the fetus is likely metabolized to the active folate forms in a more efficient manner.

The MTHFR 677TT genotype had lower cord serum folate yet higher cord RBC folate concentrations compared with the CC genotype. This observation is consistent with a recent meta-analysis comparing serum and RBC folate across MTHFR 677C>T genotypes using different assays (444). Studies utilizing protein-binding assays including the Roche Elecsys assay reported higher RBC folate in the TT compared to the CC genotype (444). However, serum folate concentrations followed the expected pattern of decreasing concentrations across genotypes (CC > CT > TT) (444). Protein-binding assays recover folate species at different affinities compared to the microbiologic assay (444). Therefore, the higher RBC folate observed in the TT genotype could be explained by the nature of the assay used and the altered folate distribution and composition associated with the T allele. Furthermore, under the conditions of high folate, the enzyme kinetics of the variant MTHFR are similar to those of the wild-type enzyme (445) and only at low folate concentrations does the functional impact of MTHFR 677C>T variant become significant (328, 329). In high cord RBC folate concentrations, therefore, the MTHFR 677C>T polymorphism may not affect RBC folate concentrations in the direction associated with diminished MTHFR activity. In contrast to higher UMFA concentrations associated with the del/del variant in adults (128), the fetal DHFR 19bp
insertion/deletion or DHFR intron 3 genotypes had no effect on cord plasma UMFA concentrations. The observed null effect may be related to the fact that UMFA did not accumulate in cord blood.

The PREFORM study provides a novel opportunity to explore the folate status of demographically diverse group of pregnant women and their newborns in Canada as well as the potential modifying effects of the fetal MTHFR and DHFR genetic variants on cord blood folate and UMFA. Comparison of our study findings to other reports in the literature is however severely limited due to significant assay-dependent and inter-laboratory differences in blood folate measurement, which have been discussed previously (332). For example, it was shown RBC folate measured by the Bio-Rad Quantaphase II immunoassay was approximately 45% lower than that determined by microbiologic assay (96-99, 439). In contrast, we showed RBC folate concentrations measured by the Immulite 2000 immunoassay were higher than those determined by the microbiologic assay (98). Thus, it is very difficult to make direct comparisons of RBC folate concentrations measured by different assays. Therefore, the observed higher RBC folate concentrations in our study might be in part due to the Roche Elecsys assay. However, in our hands, RBC folate concentrations measured by the Roche Elecsys method were on average 5.3% higher than those measured by the microbiologic assay, with all values within ± 20%. RBC folate concentration values obtained by these two different assays resulted in a Pearson correlation coefficient of 0.90 (p<0.0001). Another limitation in the present study is that folate concentrations were determined in non-fasting maternal blood samples. Fasted blood samples are shown to have lower serum folate and plasma UMFA concentrations compared with non-fasted samples (98), although RBC folate concentrations are not affected by the fasting status.
In summary, notwithstanding limitations associated with comparing RBC folate concentrations measured by different assays, maternal RBC folate concentrations were in the range generally considered to be high in a cohort of representative pregnant women in Canada with mandatory folic acid fortification and prevalent use of prenatal supplements. UMFA was detectable in >90% maternal and cord plasma samples. Both maternal and cord RBC folate and plasma UMFA concentrations were higher than the previously reported values from unfortified countries. On the positive, RBC folate concentrations were well within the range in early pregnancy (determined by the microbiologic assay) associated with protection against neural tube defects (>1000 nmol/L) (92). However, given the concerns over the potential adverse health outcomes (169), the functional ramifications of the observed high concentrations of folate and UMFA in maternal and fetal circulation in the present study warrant further investigation. Specifically, the dose of folic acid in prenatal supplements should be reconsidered in light of our findings to provide the optimal health benefits to the growing fetus while avoiding undue risk.
Chapter 6: Pyridoxal 5' phosphate concentrations in a cohort of pregnant Canadian women and umbilical cord blood


6.1 Abstract

**Background:** Vitamin B₆ is important in fetal development as an essential coenzyme involved in over 100 enzymatic reactions, including one-carbon metabolism. Little is known of the vitamin B₆ status of pregnant women and newborns in North America and potential modifying factors.

**Objectives:** This prospective study determined maternal and cord pyridoxal 5’ phosphate (PLP) plasma concentrations in a cohort of Canadian pregnant women and their newborns. The effect of maternal intake of vitamin B₆ and fetal genetic variants in one-carbon metabolism on cord plasma PLP and homocysteine concentrations was also examined.

**Design:** Dietary, supplemental, and medicinal intakes of vitamin B₆ in 368 pregnant women were assessed in early and late pregnancy. PLP concentrations were measured in maternal plasma in early pregnancy and at delivery, and in cord plasma. Six fetal variants of the *MTHFR* and *CβS* genes were assessed for their association with cord plasma PLP and homocysteine concentrations.
Results: Geometric mean (95% CI) plasma PLP concentrations were 107 (98, 116) nmol/L in early pregnancy and 58 (53, 62) nmol/L at delivery, respectively, and 296 (275, 319) nmol/L in cord blood (p<0.0001). Less than 6% of women had plasma PLP concentrations <20 nmol/L. Ninety eight percent of the women with supplemental B₆ intake of at least the RDA had plasma PLP concentrations >20 nmol/L. Plasma PLP concentrations were higher in vitamin B₆ supplement users than in nonusers in early pregnancy (p<0.0001). Fetal genetic variants did not alter cord plasma PLP and homocysteine concentrations.

Conclusions: Vitamin B₆ deficiency is uncommon in a cohort of Canadian pregnant women due largely to prevalent vitamin B₆ supplement use. Nevertheless, optimal vitamin B₆ status during pregnancy needs to be determined to ensure optimal pregnancy and birth outcomes.
6.2 Introduction

Vitamin B\textsubscript{6} is important in fetal development, in particular for nervous system development and functioning (1, 38). Vitamin B\textsubscript{6} exists naturally in three forms – pyridoxal, pyridoxine, and pyridoxamine – all of which are converted to the co-enzyme PLP (1, 38). PLP is an active coenzyme for over 100 enzymes involved in protein metabolism, hemoglobin production, and biosynthesis of neurotransmitters (1). PLP acts as a cofactor to several enzymes including C\textsubscript{beta}S, cystathionine gamma-lyase, and SHMT, which convert homocysteine to cystathionine, cystathionine to cysteine, and THF and serine to 5,10-methyleneTHF and glycine, respectively, as well as glycine decarboxylase of the glycine cleavage system that is associated with the production of one-carbon intermediates (1) (Figure 6.1). The EAR for non-pregnant adults and pregnant women is 1.1 mg/d and 1.6 mg/d, respectively, whereas the RDA is 1.3 mg/d and 1.9 mg/d, respectively. During pregnancy, low vitamin B\textsubscript{6} status has been linked to adverse pregnancy and birth outcomes (16, 17, 36), but not all studies have shown an association (18, 19). A recent systemic review of 4 randomized controlled trials (15, 446-448) has found no convincing evidence to support maternal or neonatal clinical benefits of vitamin B\textsubscript{6} supplementation during pregnancy (38).
Figure 6.1. Involvement of vitamin B<sub>6</sub> in one-carbon metabolism. CβS, cystathionine beta synthase; CTH, cystathionine gamma-lyase; DMG, dimethylglycine; MS, methionine synthase; MTHF, methyl tetrahydrofolate; MTHFD1, methylene tetrahydrofolate dehydrogenase 1; MTHFR, methylene tetrahydrofolate reductase; THF, tetrahydrofolate.

The most widely used systemic biomarker of vitamin B<sub>6</sub> status is PLP in plasma or serum since PLP reflects tissue stores of vitamin B<sub>6</sub> (449). A widely accepted cut off to indicate suboptimal PLP status is <20 nmol/L, which was used to calculate the vitamin B<sub>6</sub> EAR for women aged 19-50 y (449).

Data from the 2004 Canadian Community Health Survey 2.2 reported 13% of women aged 19-
50 y had dietary vitamin B₆ intakes below the estimated average requirement (EAR); however, this figure drops to <5% if vitamin B₆ supplementation is taken into consideration (213). The 2003-2004 NHANES data indicated that ~75% women of childbearing age using oral contraceptives had plasma PLP concentrations <20 nmol/L compared with ~13% of men of the same age and ~23% women of childbearing age not using oral contraceptives (241). More recent NHANES data from 2005-2006 found vitamin B₆ to be the most common (~11%) suboptimal micronutrient status (as indicated by PLP <20 nmol/L), with a prevalence of ~10% in females aged 20-39y (167). However, the prevalence of suboptimal vitamin B₆ status in pregnant women was not reported in this NHANES report (167).

Maternal intake and blood concentrations of vitamin B₆ during pregnancy have not been well characterized. Although several experimental and observational studies have investigated PLP concentrations in maternal and cord plasma (15, 19, 37, 192, 194, 280, 281, 450), many date back two decades and the more recent studies did not measure cord plasma PLP. Therefore, we determined maternal and cord plasma concentrations of PLP in a cohort of pregnant Canadian women and their newborns and examined the effect of maternal intake of dietary and supplemental vitamin B₆ on maternal and cord plasma concentrations of PLP. Polymorphisms in the MTHFR and CβS genes, which are integrally related to vitamin B₆ in one-carbon metabolism, have previously been shown to affect plasma PLP and homocysteine concentrations in adults (323). Therefore, we also examined the effect of six fetal single nucleotide polymorphisms (SNPs) in the MTHFR and CβS genes on cord plasma concentrations of PLP and homocysteine.
6.3 Subjects and Methods

Subjects and study design

The study design, sample size rationale, and subject characteristics have been reported in detail previously (434). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the St. Michael’s Hospital Research Ethics Board, REB 10-246. Written informed consent was obtained from all women during the initial study visit. Between November 2010 and January 2012, 368 healthy women between the ages of 18 and 45 y with an uncomplicated singleton pregnancy between 12 and 16 wk of gestation were recruited during their first visit to obstetricians to participate in the PREFORM (PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant) study (ClinicalTrials.gov identifier: NCT02244684). The aim of the PREFORM study is to determine the effects of maternal intake of folate and folic acid on DNA methylation of MNCs in cord blood. Briefly, women attending a prenatal clinic visit were recruited from obstetrician offices at St. Michael’s Hospital, a tertiary care teaching center affiliated with the University of Toronto and situated in the downtown core of Toronto, Canada. St. Michael’s Hospital serves a demographically diverse inner city population and delivers over 3000 neonates annually. At enrollment, the participants completed a Baseline Demographic and Health Questionnaire based on the Canadian Community Health Survey, Cycle 2.2 (435) to collect information regarding demographics and health status as described previously (434). Women in the study delivered between April 2011 and August 2012. At birth, the infants’ anthropometric information was recorded.
Assessment of dietary and supplemental intake

Maternal dietary intakes of vitamin B₆ and other one-carbon nutrients during early and late pregnancy were captured using a 110-item semi-quantitative Block food frequency questionnaire (FFQ; NutritionQuest, Berkeley, CA) administered during the first (12-16 wk of gestation) and second (35-37 wk of gestation) study visits. Briefly, the Block FFQ is designed to capture the consumption of specific foods and beverages in the previous three months: for the present study, consumption between 0 and 16 wk (early) and between 23 and 37 wk (mid to late) pregnancy was measured. The Block FFQ has been validated for B vitamin intake in a sample of Ontarian women of reproductive age (34-36 y) (434). The median deattenuated Pearson correlation coefficient between the Block FFQ and two 24-h recalls was reported to be 0.70 for vitamin B₆ from supplements and food (434). Vitamin supplement use was assessed for three time-periods: 30 d prior to pregnancy (preconception), conception to 16 wk of gestation (early pregnancy) and 23 to 37 wk of gestation (mid to late pregnancy) using the Baseline Demographic Questionnaire and the Block FFQ. Brand, dose and frequency of prenatal vitamins and supplements in early pregnancy were recorded. Use of the antiemetic Diclectin®, which contains 10 mg of vitamin B₆ as pyridoxine hydrochloride, during pregnancy was recorded as a binary variable (yes/no) using the Baseline Demographic Questionnaire. Vitamin B₆ supplement users in early pregnancy (0-16 wk of gestation) were characterized as those who reported taking a supplement containing vitamin B₆ and/or Diclectin® at the time of recruitment (12-16 wk of gestation). In mid to late pregnancy, information on the type and frequency, but not the dose, of vitamin B₆ supplement use was collected. Therefore, vitamin B₆ supplement use in mid to late pregnancy was recorded as a binary variable (yes/no).
Sample collection and processing

Maternal venous blood was drawn from each subject at recruitment during their first visit to obstetricians (12-16 wk of gestation) and at delivery (37-42 wk of gestation). The time of last meal and/or supplement use was recorded. The average (± SD) length of time food and/or supplements were consumed prior to blood draw was 5.2 ± 2.4 hrs. At delivery, up to 20 mL of venous blood was also obtained from the umbilical cord. Blood samples were collected in evacuated tubes containing EDTA for plasma PLP. Plasma was separated from whole blood within two hours of collection and stored at -80ºC until further analysis for plasma PLP. Approximately 8 mL of cord blood was used for MNC extraction using the ACCUSPIN System-Histopaque 1077 System (Sigma-Aldrich, St. Louis, MO, USA). Two hundred µL RNAprotect Cell Reagent (Qiagen Sciences, Germantown, MD, USA) was added to cell pellets before freezing. Cell pellets were stored at -80ºC until DNA extraction.

Plasma PLP assay

Plasma PLP concentrations were determined by A/C Diagnostics LLC (San Diego, CA, USA) using a non-radioactive enzymatic assay (239). Samples were run in 96-well plates and analyzed on the Tecan Sunrise Microplate reader (Tecan Systems, San Jose, CA, USA) at an absorbance wavelength of 675nm. The limit of detection was 10 nmol/L. Six calibrators (0, 12.5, 25.0, 100, 200 and 400 nmol/L) were used to establish linearity, and low and high controls were used for quality assurance. The inter-assay CVs (n=23) for the low and high controls were 13.4 and 9.3%, respectively. A conventionally used cut off to indicate suboptimal PLP status is <20 nmol/L, which is also the level used to calculate the vitamin B₆ EAR for women 19-50 y (1).
DNA isolation and genotyping

DNA from cord blood MNCs was isolated using the DNA Isolation Kit for Mammalian Blood (Roche Diagnostics, Mannheim, Germany). The extracted DNA was > 20 kb in all instances, was free of RNA contamination, and had an $A_{260/280}$ ratio between 1.8 and 2.0.

We examined the effects of six fetal SNPs in two genes (Figure 6.1) on cord plasma PLP and homocysteine concentrations: rs1801133 and rs1801131 in the MTHFR gene, and rs234706, rs2124459, rs2851391 and rs4920037 in the CβS gene. The MTHFR C677T (rs1801133) and CβS C699T (rs234706) SNPs have previously been shown to affect plasma PLP and homocysteine concentrations in adults (323). We included other MTHFR (rs1801131) and CβS polymorphisms (rs2124459, rs2851391 and rs4920037) based on their potential to modulate PLP or homocysteine concentrations. MTHFR catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF (1). CβS requires vitamin $B_6$ as a cofactor to convert homocysteine to cystathionine as the first irreversible step in the transsulfuration pathway (1). The MTHFR polymorphisms were genotyped using the TaqMan genotyping assay on an ABI Vii7 Biosystem (Applied Biosystems, Foster City, CA, USA) as described previously (437). The CβS polymorphisms were genotyped using the iPLEX Assay with the MassARRAY platform (Sequenom, San Diego, CA, USA) at the Clinical Genomics Centre (Toronto, Canada) as described previously (438).

Statistical analysis

Maternal and cord plasma PLP concentrations were log transformed to meet the assumptions of normality. Geometric mean with corresponding 95% CI was reported. Differences in plasma PLP concentrations at three time points (maternal plasma at recruitment, maternal plasma at delivery,
and cord plasma) were analyzed using generalized estimating equations (PROC GENMOD) in order to take repeated measures into account. Student’s t-test was used to determine differences between vitamin B<sub>6</sub> supplement users and nonusers. Correlations between dietary and blood parameters were assessed using Pearson’s tests. To determine if changes in PLP concentration across pregnancy was dependent on maternal PLP concentration at 12-16 wk gestation, the difference between PLP concentrations at delivery and at 12-16 wk gestation was calculated. The nonparametric Wilcoxon test was used since the differences were skewed. For this analysis, early pregnancy PLP concentrations were dichotomized as <20 and ≥20 nmol/L. Least squares regression and generalized least squares regressions were used to identify predictors of maternal and cord plasma PLP. Potential predictors for maternal plasma PLP concentrations included maternal age, BMI, education level, ethnicity, Diclectin® use, vitamin B<sub>6</sub> intake and supplemental vitamin B<sub>6</sub> intake in early pregnancy. Potential predictors for cord plasma PLP concentrations included maternal age, BMI, educational level, ethnicity, dietary vitamin B<sub>6</sub> intake in late pregnancy, maternal PLP concentrations at delivery, and concentrations of other nutrients involved in one-carbon metabolism (serum folate and vitamin B<sub>12</sub>, plasma homocysteine). Covariates that were significantly associated with the outcome variable (p<0.05) were retained in the final model. An exploratory analysis was conducted to examine the effects of the selected fetal genotypes on cord plasma PLP concentrations. Differences in cord plasma PLP concentrations (outcome variables) across fetal genotype (explanatory variables) were assessed with the use of least squares regression (PROC GLM). Unadjusted models for differences in cord plasma PLP concentrations among fetal genotypes were reported.

In these analyses, maternal plasma samples (n=17) collected at delivery and cord plasma samples (n=12 for PLP, n=14 for homocysteine) were excluded from infants born <37 wk gestation
(preterm) (n=20). All tests were two-tailed and an alpha level < 0.05 was considered statistically significant. Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). All available data was included in analyses and the samples size for each analysis was reported.
6.4 Results

Characteristics of the study subjects and newborn infants

Detailed information on the PREFORM study design and characteristics of the study participants has been published previously (434). Of 1,315 pregnant women approached, 906 met eligibility criteria (healthy, 18-45 y of age, uncomplicated singleton pregnancy, <16 wk post-conception at the time of enrolment). Women were excluded if they were taking medications known to interfere with folate metabolism, planned to deliver at another hospital, or planned to bank umbilical cord blood. After applying these exclusion criteria, 368 pregnant women enrolled in the study and 309 deliveries occurred (Figure 6.2). Maternal and newborn characteristics from the PREFORM Study have been published elsewhere (434). Briefly, the PREFORM study consisted of primarily nulliparous (54%), Caucasian (45%), university educated (62%) pregnant women, with the mean (± SD) age of 32 ± 5 y. The mean (±SD) newborn gestational age was 39.3 ± 1.7 wk. Approximately 85% of women who completed the early pregnancy visit remained in the study at delivery. The primary reason given for not continuing in the study was transfer or delivery at another hospital or moved away (n=24). Furthermore, maternal plasma samples (n=17) collected at delivery and cord plasma samples (n=12 for PLP, n=14 for homocysteine) were excluded from infants born <37 wk of gestation (preterm) (n=20).

Birth weight, length and head circumference were 3346 ± 540 g, 52 ± 3 cm, and 34.2 ± 1.8 cm, respectively. There were no differences in gestational age at birth, sex of infant, birth weight, birth length or head circumference between newborns from mothers who used B-vitamin containing supplements throughout pregnancy and those from mothers who did not (451).
Figure 6.2. Flowchart of PLP and homocysteine sample collection. GW, gestational weeks.
Dietary, supplemental and medicinal intakes of vitamin $B_6$

Vitamin $B_6$ intakes and plasma concentrations of PLP in the PREFORM Study are summarized in Figure 6.3. Detailed information on dietary and supplemental intakes of vitamin $B_6$ and other one-carbon nutrients of the PREFORM study participants has been published previously (434). Briefly, the mean ($\pm$ SD) maternal dietary vitamin $B_6$ intakes were slightly higher than the EAR (1.6 mg/d) in early (1.8 ± 0.9 mg/d) and mid to late (1.7 ± 0.7 mg/d) pregnancy (434). Eighty-seven percent of the women reported using a vitamin $B_6$-containing supplement in early pregnancy, mostly in the form of a prenatal multivitamin formulation containing 1.9 mg of vitamin $B_6$. Ninety-one percent and 0.3% women had supplemental vitamin $B_6$ intakes at the recommended dietary allowance (RDA: 1.9 mg/d) and tolerable upper limit (100 mg/d), respectively, in early pregnancy (434). In addition, 32 (8.7%) women reported taking Diclectin® in early pregnancy to reduce nausea. Self-reported supplemental vitamin use in mid to late pregnancy was only reported as a categorical variable (yes/no) and hence, no supplemental vitamin $B_6$ intake dosages were reported for late pregnancy.
Figure 6.3. Schematic of vitamin B₆ intakes and plasma PLP concentrations in the PREFORM Study.

Maternal and cord plasma PLP concentrations

Geometric mean (95% CI) PLP concentrations in maternal plasma in early pregnancy (12-16 wk) and at delivery (37-42 wk), and cord plasma are presented in Table 6.1. During early pregnancy and at delivery, 3.6 and 5.5% of women had plasma PLP concentrations <20 nmol/L, respectively. Women with plasma PLP <20 nmol/L in early pregnancy did not differ in ethnicity (p=0.9), early pregnancy BMI (p=0.9), or education level (p=0.07), but were younger [mean (95% CI): 28 (25, 31) y] compared to those with plasma PLP ≥20 nmol/L [32 (31-32) y] (p=0.004). Furthermore, of women with plasma PLP <20 nmol/L in early pregnancy, 73% were vitamin B₆ supplement users, one participant reported Diclectin® use, and 55% were smokers. Ninety three percent of women with plasma PLP <20 nmol/L in late pregnancy were vitamin B₆ supplement users and 89% reported smoking in late pregnancy.
Table 6.1. Maternal and cord plasma pyridoxal 5’ phosphate (PLP) concentrations

<table>
<thead>
<tr>
<th>Sample size</th>
<th>PLP concentrations, nmol/L</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; supplement users*</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; supplement nonusers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal plasma at 12-16 wk gestation&lt;sup&gt;1&lt;/sup&gt;</td>
<td>340</td>
<td>107 (98, 116)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110 (100, 120)</td>
<td>71 (48, 102)</td>
</tr>
<tr>
<td>Maternal plasma at delivery</td>
<td>279</td>
<td>58 (53, 62)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59 (54, 63)</td>
<td>55 (44, 68)</td>
</tr>
<tr>
<td>Cord plasma</td>
<td>235</td>
<td>296 (275, 319)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>307 (282, 333)</td>
<td>233 (191, 284)</td>
</tr>
</tbody>
</table>

Geometric mean (back-transformed 95% CI) reported. T-tests were used to compare supplement users versus nonusers; p-value testing the null hypothesis of no difference between users and nonusers. In the column describing all subjects, values with different superscript letters were significantly different from corresponding values at different time points (all p<0.0001). *For maternal concentrations at 12-16 wk gestation, supplement use was during early pregnancy and/or use of Diclectin®; for maternal concentrations at delivery and cord blood concentrations, supplement use was during late pregnancy. <sup>1</sup>Participant recruitment occurred at 12-16 wk gestation when maternal blood was collected.

Overall, maternal plasma PLP concentrations decreased by 45% during pregnancy (p<0.0001). Interestingly, for mothers with plasma PLP concentrations <20 nmol/L at 12-16 wk gestation, their plasma PLP concentrations at the time of delivery increased by 16 (11, 32) [median (25<sup>th</sup>, 75<sup>th</sup> percentile)] nmol/L. Conversely, for mothers with PLP concentration ≥20 nmol/L at 12-16 wk gestation, their plasma PLP concentrations decreased by 54 (14, 108) [median (25<sup>th</sup>, 75<sup>th</sup> percentile)] nmol/L. Cord plasma PLP concentrations were approximately 5 times maternal plasma PLP concentrations at delivery (p<0.0001).
Plasma PLP concentrations during early pregnancy differed significantly between those who took supplemental and/or medicinal vitamin B<sub>6</sub> and those who did not (p<0.0001). In contrast, at delivery maternal plasma PLP concentrations did not differ between vitamin B<sub>6</sub> supplement users and nonusers (p=0.7). Plasma PLP concentrations were significantly higher in cord blood from mothers who took vitamin B<sub>6</sub> supplements in late pregnancy than in cord blood from nonusers (p=0.03). Ninety-eight percent of the women with vitamin B<sub>6</sub> supplemental intake of at least the RDA met or exceeded plasma PLP concentrations of 20 nmol/L.

We have previously reported homocysteine concentrations in maternal plasma in early pregnancy and at delivery, and cord plasma from the PREFORM study (451). In maternal plasma, PLP concentrations were negatively correlated with homocysteine concentrations at delivery (r = -0.15, p = 0.01) but not in early pregnancy. In cord plasma, PLP concentrations were weakly inversely correlated with homocysteine concentrations (r = -0.13, p = 0.06).

**Association of maternal dietary and supplemental vitamin B<sub>6</sub> intakes with plasma PLP concentrations**

Maternal dietary intakes of vitamin B<sub>6</sub> were not associated with maternal PLP concentrations either in early pregnancy, at delivery or in cord plasma (data not shown). In unadjusted general linear models, supplemental vitamin B<sub>6</sub> use in early pregnancy predicted maternal PLP concentrations during early pregnancy (β [95% CI]: 3.2 [2.5, 4.0]) (p<0.0001). This relationship remained significant after adjusting for covariates ([95% CI]: 4.6 [3.7, 5.6]) (p <0.0001). For every 1 mg/d increase in maternal supplemental vitamin B<sub>6</sub>, an approximate average increase of 5 nmol/L in maternal plasma PLP concentrations is expected.
Association between maternal and cord plasma PLP concentrations

Maternal plasma PLP concentrations during early pregnancy were correlated with maternal plasma PLP concentrations at delivery and cord plasma PLP concentrations (both r=0.34, p<0.0001). In unadjusted general linear models, maternal plasma PLP concentrations at delivery were positively associated with cord plasma PLP concentrations (β [95% CI]: 2.5 [2.1, 2.8]) (p<0.0001). Adjusting for additional covariates did not change the association (β [95% CI]: 2.2 [1.9, 2.6]) (p<0.0001). For every 1 nmol/L increase in maternal plasma PLP concentrations, an approximate average increase of 2 nmol/L in cord plasma PLP concentrations is expected.

Cord plasma PLP concentrations based on infant genotype

Associations between six fetal SNPs in two genes, which are integrally related to vitamin B₆ in one-carbon metabolism and which can potentially affect plasma PLP and homocysteine concentrations, and cord plasma PLP and homocysteine concentrations were examined. All genotypes except CβS 699C>T (rs234706; p=0.03) were found to be in Hardy-Weinberg equilibrium (p>0.16), indicating the observed genotype frequencies were not different from that expected. Minor allele frequency for all genotypes was >5% (MTHFR rs1801133, 29.9%; MTHFR rs1801131, 30.5%; CβS rs234706 23.5%, CβS rs2124459 47.9%, CβS rs2851391 43.0%, CβS rs4920037 16.1%). None of the fetal SNPs was significantly associated with cord plasma PLP or homocysteine concentrations (Table 6.2).
Table 6.2. Plasma PLP concentrations in umbilical cord blood according to fetal genotype¹

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Genotype Frequency, (%)</th>
<th>Cord PLP, nmol/L</th>
<th>p-value</th>
<th>Cord plasma homocysteine, µmol/L</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>51</td>
<td>361 (326, 397)</td>
<td>0.48</td>
<td>4.9 (4.6, 5.3)</td>
<td>0.66</td>
</tr>
<tr>
<td>MTHFR</td>
<td>rs1801133</td>
<td>CT</td>
<td>40</td>
<td>339 (295, 382)</td>
<td></td>
<td>5.1 (4.8, 5.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>9</td>
<td>322 (228, 415)</td>
<td></td>
<td>5.0 (4.4, 5.6)</td>
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</tr>
<tr>
<td>MTHFR</td>
<td>rs1801131</td>
<td>AA</td>
<td>50</td>
<td>360 (322, 397)</td>
<td>0.37</td>
<td>4.9 (4.6, 5.2)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC</td>
<td>39</td>
<td>361 (319, 402)</td>
<td></td>
<td>5.1 (4.8, 5.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>11</td>
<td>298 (223, 373)</td>
<td></td>
<td>5.1 (4.6, 5.7)</td>
<td></td>
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<tr>
<td>CβS</td>
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<td>7</td>
<td>352 (296, 408)</td>
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<td>5.1 (4.5, 5.8)</td>
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<tr>
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<td></td>
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<td>339 (300, 377)</td>
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<tr>
<td></td>
<td></td>
<td>TT</td>
<td>61</td>
<td>365 (319, 411)</td>
<td></td>
<td>4.8 (4.6, 5.1)</td>
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<tr>
<td>CβS</td>
<td>rs2124459</td>
<td>AA</td>
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<td>4.6 (4.3, 5.0)</td>
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<td></td>
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<td>47</td>
<td>345 (297, 393)</td>
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<td>5.0 (4.8, 5.3)</td>
<td></td>
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<tr>
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<td></td>
<td>GG</td>
<td>32</td>
<td>359 (325, 393)</td>
<td></td>
<td>4.9 (4.5, 5.2)</td>
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<tr>
<td>CβS</td>
<td>rs2851391</td>
<td>CC</td>
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<td>0.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
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<td>343 (304, 381)</td>
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<td>5.0 (4.7, 5.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
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<td>355 (297, 414)</td>
<td></td>
<td>4.9 (4.5, 5.4)</td>
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<tr>
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<td>72</td>
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<td>5.0 (4.0, 6.0)</td>
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<tr>
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<td></td>
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<td></td>
<td>5.1 (4.7, 5.4)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>3</td>
<td>356 (325, 386)</td>
<td></td>
<td>4.8 (4.6, 5.1)</td>
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</tr>
</tbody>
</table>

¹Values shown are means (95% CI) for cord PLP concentration. Differences between genotypes were assessed using least squares regression with fetal genotype as the explanatory variable and cord plasma PLP or homocysteine concentrations as the outcome variable. CβS, cystathionine beta synthase; MTHFR, methylene tetrahydrofolate reductase.
6.5 Discussion

In a cohort of demographically diverse pregnant women receiving prenatal care in Toronto, Ontario, >85% of whom were taking a periconceptional vitamin B₆-containing supplement, with the most common dose of 1.9 mg/d, only <6% had plasma PLP concentrations <20 nmol/L during pregnancy, a commonly used cutoff for low vitamin B₆ status (1). Previous observational studies have reported both larger (16% in Singapore) (19, 194) and smaller (0% and 0.2% in 1st and 2nd trimesters, respectively, in Canada) (192) proportions of pregnant women with plasma PLP concentrations <20 nmol/L. Although <20 nmol/L is generally used as the suboptimal PLP cutoff, others have suggested <30 nmol/L to indicate suboptimal PLP (1). In our cohort, 6.8% and 14.3% of women had plasma PLP <30 nmol/L in early pregnancy and at delivery, respectively. A study from Japan found 16%, 82%, and 84% of pregnant women had plasma PLP concentrations <30 nmol/L in the 1st, 2nd and 3rd trimesters, respectively (279). This is not surprising considering these women were not taking supplements and their dietary vitamin B₆ intakes were considerably low (<1.0 mg/d) (279). Another Japanese study reported 100% of pregnant women had plasma PLP concentrations <30 nmol/L, again likely relating to lower vitamin B₆ intake compared with North America (194).

The functional and clinical significance of the cutoff plasma PLP concentration for low vitamin B₆ status still remains to be clarified (452). Metabolic balance studies involving subjects intravenously supplemented with vitamin B₆ for 4 weeks determined health risks such as seborrheic dermatitis, microcytic anemia, and epileptiform convulsions (1), were not apparent in subjects with plasma PLP concentrations > 20 nmol/L (1, 453). However, it is still unknown whether it is optimal to maintain plasma PLP concentrations >20 nmol/L during pregnancy.
Few clinical studies have suggested that high vitamin B₆ status during pregnancy is associated with decreased prenatal nausea and vomiting (454-456), higher Apgar scores (15, 447), higher birth weights (37), reduced incidence of pre-eclampsia (457) and preterm birth (16), and a protection against orofacial clefts (458) and cardiovascular malformations (38, 291). However, these purported associations have not unequivocally been demonstrated (18, 19, 38).

Maternal plasma PLP concentrations decreased from early to late pregnancy in our study, which is consistent with previous observations (192, 194, 279). Interestingly, there were distinct opposing trends across pregnancy depending on the mother’s plasma PLP status in early pregnancy: Plasma PLP concentrations increased during pregnancy in those with plasma PLP concentrations <20 nmol/L in early pregnancy, while the opposite trend was observed in those with plasma PLP concentrations ≥20 nmol/L in early pregnancy. Our overall observation of decreasing plasma PLP during pregnancy is likely related to normal physiological changes associated with pregnancy rather than diminished vitamin B₆ status. Plasma PLP concentrations are known to decrease during pregnancy likely due to increased plasma volume (1) and fetal sequestering of PLP for rapid fetal growth and development (452). The observed decrease in our study is unlikely related solely to hemodilution as mean (± SD) maternal hematocrit did not significantly change between early pregnancy [(0.36 ± 0.03 L/L; range 0.29-0.42 L/L) and delivery (0.36 ± 0.03 L/L; range 0.26-0.42 L/L), p=0.77]. Experimental studies in pregnancy have shown that supplemental intakes of 4-10 mg/d pyridoxine hydrochloride are needed to maintain plasma PLP concentrations at pre-pregnancy or first trimester levels (15, 37, 450). Approximately 10% of the participants reported using Diclectin® containing 10 mg pyridoxine hydrochloride during early pregnancy to reduce pregnancy-associated nausea. Most of these women stopped using Diclectin® after the first trimester as their nausea improved. This might
have contributed to the observed decrease in plasma PLP concentrations during pregnancy. Functional ramifications of the decrease in plasma PLP during pregnancy are uncertain. Only <6% of the women had PLP concentrations <20 nmol/L at delivery despite the decline and no women had elevated homocysteine concentrations (>13 μmol/L), a nonspecific inverse indicator of vitamin B₆ deficiency (1) (Figure 6.1).

Adequate dietary intakes and prevalent supplemental use of vitamin B₆ in the present study likely contributed to higher plasma PLP concentrations compared with previous studies from other countries (19, 194, 280, 281). However, different assays (HPLC and enzymatic) employed across the published studies and limitations associated with these assays preclude direct comparison of our data with others (240). Furthermore, fortification practices might have contributed to the discrepancy. In Canada, the addition of 0.31 mg vitamin B₆ per 100 g flour and 0.6 mg per 100 g of breakfast cereal is an optional and yet widespread fortification practice (282, 459). In our study cohort, breakfast cereals were the highest ranked fortified food contributing to total vitamin B₆ intake in early pregnancy (434). Indeed, a recent Canadian study has reported comparable maternal PLP concentrations [median (95%CI) 94 (82, 112 nmol/L) and 76 (70, 83 nmol/L) in first and second trimesters, respectively] to the present study (192). Another contributing factor is the use of Diclectin® (Diclegis® in the United States), which contains 10 mg pyridoxine hydrochloride (287). Users following the standard recommended dose (290) would consume up to 40 mg of pyridoxine hydrochloride per day. In our study, Diclectin® use was a significant predictor of maternal plasma PLP concentrations at 12-16 wk of gestation. Also, when Diclectin® use was not accounted for, the significant difference in maternal PLP concentrations between supplement users and nonusers disappeared. These observations suggest that Diclectin® had an appreciable effect on maternal plasma PLP concentrations.
Similar to a previous study (193), cord plasma PLP concentrations were five times the maternal plasma PLP concentrations at delivery. A small observational study (n=31) from Germany reported much lower cord plasma PLP concentrations (mean ± SD: 64 ± 39 nmol/L) compared with the present study (281). Three clinical trials have reported that maternal vitamin B₆ supplementation increases cord plasma PLP concentrations in a dose-dependent manner (15, 37, 450). These studies have also observed that PLP concentrations are higher in cord plasma than in maternal plasma at delivery (15, 37, 450). Placental perfusion studies have shown vitamin B₆ is converted to its active form and then actively transferred to the fetus (460, 461).

None of the six fetal genotypes influenced cord plasma PLP concentrations in our population. In a large-scale Norwegian study of adults, the T variant in the MTHFR C677T SNP was associated with a significant increase in plasma homocysteine and a decrease in plasma PLP (323). The CβS 699C>T polymorphism was negatively associated with plasma homocysteine concentrations (323). Hormonal influences, hemodilution, and increased fetal demand for PLP may exert a stronger influence on PLP and homocysteine in cord plasma and may explain why we failed to see an association. In contrast to these null findings, maternal plasma PLP concentrations at delivery were significantly associated with cord plasma PLP concentrations. Together, these data suggest that maternal plasma PLP concentrations exert a greater influence on cord plasma PLP concentrations than fetal genotype at the time of birth.

The PREFORM study is a large comprehensive observational study assessing maternal and cord plasma vitamin B₆ status in a demographically diverse group of pregnant North American women and their infants. This is the first study to assess the potential modifying effects of maternal dietary and supplemental intake of vitamin B₆ including an important source of medicinal vitamin B₆ (Diclectin®) and fetal genotypes on cord plasma PLP concentrations.
However, there are several limitations associated with our study. Although we examined two fetal genotypes in our analysis, we did not interrogate other genetic variants in several vitamin B$_6$ dependent enzymes such as serine hydroxymethyltransferase and other enzymes known to affect PLP concentrations such as alkaline phosphatase and aldehyde oxidase (343, 462, 463). Furthermore, maternal DNA was not available for genotyping. Because maternal genotypes may alter metabolism of vitamin B$_6$ dependent metabolic pathways and thus, maternal plasma PLP concentrations, maternal genotype data would have strengthened our understanding of factors that influence maternal and cord plasma PLP concentrations. Plasma PLP concentrations were determined in non-fasting maternal blood samples. As vitamin B$_6$ and protein intake (464, 465) can influence PLP concentrations, standardized collection of fasting blood samples would have been more ideal. Because study subjects were recruited and the initial blood was drawn from those agreeing to participate during their first visit to obstetricians, we could not limit the recruitment to those who had been fasting. Also, as blood was taken from the participants when they presented for delivery, we could not enforce the fasting state. However, we recorded the time of last meal and/or supplement use in early pregnancy and the average (± SD) length of time food and/or supplements were consumed prior to blood draw was 5.2 ± 2.4 hrs. A sensitivity analysis showed that plasma PLP concentrations were not significantly different between those who had blood taken < 5 hours and those who had blood taken > 5 hours after their last meal or supplemental intake (p=0.42). The same relationship was found using 8 hour and 12 hour fasting as cutoffs (p=0.19 and p=0.07, respectively). However, we cannot rule out a possibility that there might be a higher proportion of women with plasma PLP concentrations > 20 nmol/L relating to the transient increase in PLP concentrations following vitamin B$_6$ intake from food or supplements because we measured PLP concentrations in a non-fasting state. We
did not measure other functional indicators of vitamin B\textsubscript{6} status such as transaminase activity or kynurenines concentrations in the present study (463). However, the measurements of these indicators are not widely available.

In the present study, 94\% of pregnant Canadian women had plasma PLP concentrations $\geq$20 nmol/L. The EAR for vitamin B\textsubscript{6} during pregnancy is 1.6 mg/d (1), corresponding to the median intake of the PREFORM study participants in early and late pregnancy (434). This suggests that inadequate vitamin B\textsubscript{6} intakes, from dietary sources only, in at least one-half of the PREFORM study participants (434). However, 91\% of the pregnant Canadian women met the RDA through B\textsubscript{6} supplement use alone. Ninety eight percent of the women with vitamin B\textsubscript{6} supplemental intake of at least the RDA had plasma PLP concentrations $\geq$ 20 nmol/L. Therefore, vitamin B\textsubscript{6} supplements appear to be an important source of vitamin B\textsubscript{6} to ensure adequate maternal PLP concentrations during pregnancy, but not all women are achieving optimal PLP concentrations. The effects of prenatal vitamin B\textsubscript{6} status on pregnancy and birth outcomes and on health outcomes of the offspring have not yet been clearly established. Therefore, optimal vitamin B\textsubscript{6} status during pregnancy should be determined to ensure balance between the health benefits and risks to the growing fetus.
Chapter 7: Effects of maternal one-carbon nutrient status on global DNA methylation and hydroxymethylation in cord blood mononuclear cells


7.1 Abstract

**Background:** DNA methylation of the developing fetus is susceptible to environmental modifiers including maternal diet. Aberrant or disturbed DNA programming during embryogenesis may have permanent phenotypic and functional effects in the offspring and may confer disease susceptibility later in life. Given the critical role of one-carbon nutrients in biological methylations including DNA methylation, we determined whether maternal status of these nutrients affects DNA methylation in the human offspring.

**Objectives:** This prospective study involving a cohort of pregnant Canadian women and their newborn infants examined the association of maternal and cord blood concentrations of one-carbon nutrients and their metabolites and biomarkers with the total content of 5-methylcytosine and 5-hydroxymethylcytosine, measures of global DNA methylation and demethylation, respectively, in cord blood mononuclear cells.
**Design:** Serum and RBC folate, plasma unmetabolized folic acid, serum vitamin B\(_{12}\), and, methylmalonic acid, plasma pyridoxal 5’ phosphate, plasma choline, betaine, dimethylglycine, trimethylamine oxide, and plasma homocysteine were measured in a cohort of 368 pregnant women in early pregnancy (12-16 wks) and at delivery (37-42 wks), and in cord blood. Total 5-methylcytosine and 5-hydroxymethylcytosine content in DNA was quantified by LC-MS/MS in cord blood mononuclear cells. The potential modifying effects of 38 fetal genetic variants in 26 genes involved in one-carbon metabolism in cord blood mononuclear cells were also examined.

**Results:** Maternal serum vitamin B\(_{12}\) concentrations in early pregnancy were positively correlated with 5-methylcytosine content in cord blood mononuclear cells (r=0.25, p=0.002). Plasma betaine (r=0.20, p=0.01) concentrations in early pregnancy were positively, whereas early pregnancy maternal RBC folate (r=-0.16, p=0.04) and cord plasma unmetabolized folic acid (r=-0.23, p=0.004) concentrations were negatively, correlated with 5-hydroxymethylcytosine content. Two polymorphisms in the fetal 5-methyltetrahydrofolate homocysteine methyltransferase (p =0.04) and γ-glutamyl hydrolase (p=0.003) genes were associated with 5-methylcytosine content in cord blood mononuclear cells. However, these associations were not significant after controlling for multiple tests. Interestingly, infants born to mothers with the highest quartile of RBC folate concentrations and the lowest serum vitamin B\(_{12}\) concentrations in early pregnancy had significantly lower 5-methylcytosine content compared to mothers with lower RBC folate and higher serum vitamin B\(_{12}\) concentrations (p=0.007).

**Conclusions:** After adjustment for multiple testing, no associations were found between maternal or fetal concentrations of one-carbon nutrients and their metabolites and biomarkers and global DNA methylation or hydroxymethylation in fetal mononuclear cells. Furthermore, no significant effect of fetal genetic variants of one-carbon metabolism on fetal DNA methylation
and hydroxymethylation was observed. However, the observed association between high RBC folate and low serum vitamin B_{12} concentrations in maternal blood in early pregnancy and fetal DNA methylation warrants confirmation and its functional ramifications need to be determined.
7.2 Introduction

Folate, choline, vitamin B₆ and vitamin B₁₂ are important nutrients involved in one-carbon metabolism essential for biological methylation reactions including DNA methylation (Figure 7.1) (1). Folate, in the form of 5-methylTHF, donates a methyl group to homocysteine for the regeneration of methionine, which is the precursor of SAM, the primary methyl group donor for most biological reactions including DNA methylation (395). Vitamin B₁₂ functions as the coenzyme of MS, which catalyzes the transfer of a methyl group from 5-methylTHF to remethylate homocysteine to methionine (46). Choline is irreversibly oxidized to betaine, which transfers a methyl group to homocysteine for methionine regeneration catalyzed by BHMT (1). Vitamin B₆ is converted to the co-enzyme PLP, which acts as a co-factor to the three enzymes involved in transsulfuration (CβS and cystathionine γ-lyase) necessary for homocysteine clearance and SHMT, which converts THF and serine to 5,10-methyleneTHF and glycine, respectively (1). Diets deficient in and supplementation of different combinations of these one-carbon nutrients have been shown to influence DNA methylation in animal and human studies (reviewed in (379, 395)).
Figure 7.1: The methylation pathway involving one-carbon nutrients.

BER, base excision repair protein; -CH₂-THF, -methylene tetrahydrofolate; -CH₃, methyl group; -CH₂OH, hydroxymethyl group; CBS, cystathionine beta synthase; DHFR, dihydrofolate reductase; DMG, dimethylglycine; Hcy, homocysteine; Met, methionine; -MTHF, methyl tetrahydrofolate; MTHFR, methylene tetrahydrofolate reductase; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; TDG, thymine DNA glycosylase; TET, ten eleven translocation proteins; THF, tetrahydrofolate.

DNA methylation of cytosine in the cytosine-guanine dinucleotides sequences (CpG) is an important epigenetic determinant in gene expression (generally an inverse relationship) and
genomic stability (358, 372, 466). Aberrant patterns and dysregulation of DNA methylation are associated with the development of several human diseases including asthma (373), cancer (5, 395), cardiovascular disease (375), and metabolic disorders (376, 377). DNA methylation is a dynamic process between active methylation, mediated by CpG DNA methyltransferases using SAM as the methyl donor (358), and removal of methyl groups from 5mC by several mechanisms, including active demethylation by demethylases MeCP2 and MBD2 (358). 5mC can be actively converted to 5hmC, which can subsequently be converted back to an unmodified cytosine (366). Therefore, 5hmC, which is present in mammalian DNA at a significant level in a tissue-specific manner, appears to be an intermediate in DNA demethylation (358, 366). Growing evidence suggests 5hmC has its own independent effect on gene transcription by recruiting or inhibiting DNA binding proteins (358).

DNA methylation patterns and levels of 5mC and 5hmC are programmed during embryogenesis by genome-wide demethylation after fertilization, which erases large parts of the parental DNA methylation, followed by de novo methylation mostly limited to non-CpG island areas, soon after implantation (358, 381). This programming establishes a new DNA methylation pattern in the offspring, which is maintained postnatally (358, 381). DNA methylation of the developing fetus is highly susceptible to environmental modifiers including maternal diet. Indeed, maternal supplementation of one-carbon nutrients including folic acid (the synthetic form of folate), vitamin B_{12}, choline and betaine during pregnancy has been shown to change offspring’s coat color and straightness of the tail via CpG DNA methylation at the regulatory region of the A^vy and Axin^FU genes, respectively, in the agouti viable yellow and AxinFused mouse models (393, 394, 467). In agouti mice, changes in DNA methylation were associated with altered disease susceptibility, including adiposity, hyperinsulinemia, and cancer (467). The effect of maternal
supplementation of individual one-carbon nutrients on DNA methylation in the offspring has not been well studied except for folic acid. Maternal folic acid supplementation appears to alter global and gene-specific DNA methylation in the offspring in animal studies (79, 80, 397, 405, 406, 468). Recent human studies have provided limited support, albeit equivocal, for the modulatory effect of periconceptional folic acid supplementation on global and gene-specific DNA methylation in the cord blood lymphocytes (414-420, 423, 424).

Intrauterine exposure of the developing fetus to folate and folic acid has significantly increased in North America owing to mandatory folic acid fortification and periconceptional supplementation aimed at reducing the risk of neural tube defects (101, 103, 107, 163, 469). Furthermore, as requirements for vitamin B₁₂, vitamin B₆, and choline are increased during pregnancy to facilitate adequate growth and development of fetal and maternal tissues (1), women of childbearing age and pregnant women are recommended to increase dietary intakes of these one-carbon nutrients, which is often achieved through periconceptional and prenatal supplement use (189, 191). Concerns for potential adverse effects of the increased maternal dietary intakes and blood levels of one-carbon nutrients on health outcomes of the offspring in part through alterations in DNA methylation have begun to emerge. In this regard, periconceptional high folic acid intake has been linked to aberrant embryonic development in mice (32, 34, 470, 471) and humans (472), increased risks of asthma and respiratory tract infections (7, 473, 474), and increased risk of mammary tumors in rats (79) but decreased risk of colon cancer in rats (80) and several pediatric cancers in humans (197, 293). The combination of high maternal folic acid and low vitamin B₁₂ status has also been associated with small-for-gestational-age babies and obesity and insulin resistance in the human offspring (6, 475).
In the present study, we determined the association of maternal and cord blood concentrations of folate, vitamin B\textsubscript{12}, vitamin B\textsubscript{6} and choline with total content of 5mC and 5hmC in cord MNCs in a cohort of pregnant Canadian women and their newborns. Furthermore, we examined potential modifying effects of 38 fetal genetic variants in 26 genes encoding key enzymes involved in one-carbon metabolism and biological methylations on the total content of 5mC and 5hmC in cord blood MNCs.
7.3 Subjects and Methods

Subjects and study design

All procedures and protocols were reviewed and approved by St. Michael’s Hospital (Toronto, Canada) Research Ethics Board, and written informed consent was obtained from all women during the initial study visit. Between November 2010 and January 2012, 368 healthy women between the ages of 18 and 45 years with an uncomplicated singleton pregnancy between 12 and 16 weeks of gestation were recruited to participate in the PREFORM (PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant) study (ClinicalTrials.gov identifier: NCT02244684). The PREFORM study design and subject characteristics have been reported in detail previously (434). Briefly, women attending a prenatal clinic visit were recruited from obstetrician offices at St. Michael’s Hospital, situated in the downtown core of Toronto, Canada. St. Michael’s Hospital serves a demographically diverse inner city population and delivers over 3000 neonates annually. At enrollment, the participants completed a Baseline Demographic and Health Questionnaire based on the Canadian Community Health Survey, Cycle 2.2 (435) to collect information regarding demographics and health status as described previously (434). Women participating in the study delivered between April 2011 and August 2012. At birth, the infants’ anthropometric information was recorded.

Assessment of dietary and supplemental intake

Maternal dietary intakes of one-carbon nutrients during early and late pregnancy were captured using a 110-item semi-quantitative Block food frequency questionnaire (FFQ; NutritionQuest, Berkeley, CA) administered during the first (12-16 wk of gestation) and second (35-37 wk of
gestation) study visits as described previously (434). Briefly, the Block FFQ is designed to capture the consumption of specific foods and beverages in the previous three months and in this study between 0 and 16 wk (early) and 23 and 37 wk (late) pregnancy. The Block FFQ has been validated for B vitamin intake in a sample of Ontarian women of reproductive age (34-36 y) (430). The median deattenuated Pearson correlation coefficients between the Block FFQ and two 24-h recalls was reported to be 0.76 for folate, 0.70 for vitamin B₆, and 0.65 for vitamin B₁₂ from supplements and food (430). Although not validated for choline and betaine, the Block FFQ has been used previously to assess intake of these nutrients (21, 300). Vitamin supplement use was assessed over three time-periods: pre-conception (30 days prior to pregnancy), early pregnancy (conception to 16 wk gestation) and late pregnancy (23 to 37 wk gestation) using sections of the Canadian Community Health Survey, cycle 2.2 and the Block FFQ as described previously (434). The brand, dose and frequency of prenatal vitamins and supplements use preconception and early pregnancy were recorded. In late pregnancy, information on the type and frequency, but not the dose, of vitamin supplement use was recorded as a binary variable (yes/no).

**Sample collection and processing**

Maternal venous blood was drawn from each subject at study recruitment (12-16 wk of gestation) and at delivery (37-42 wk of gestation). The participants were not instructed to fast prior to blood collection and the time of last meal and/or supplement use was recorded. The average (± SD) length of time food and/or supplements were consumed prior to blood draw was 5.2 ± 2.4 hrs. At delivery, up to 20 mL of venous blood was also obtained from the umbilical cord. Blood samples were collected in evacuated tubes containing EDTA for plasma and RBC analysis or those containing coagulant and gel for serum separation. Plasma and serum were isolated from whole blood within two hours of collection and serum and RBC folate, serum
vitamin B₁₂ and plasma homocysteine concentrations were determined immediately in the St. Michael’s Hospital Core Laboratory. The remaining plasma was stored at -80°C until further analysis for plasma UMFA, MMA, PLP, choline, betaine, DMG, and TMAO concentrations. Approximately 8 ml of cord blood was used for MNC extraction using the ACCUSPIN System-Histopaque 1077 System (Sigma-Aldrich, St. Louis, MO, USA). Two hundred µl RNAprotect Cell Reagent (Qiagen Sciences, Germantown, MD, USA) was added to cell pellets before freezing. Cell pellets were stored at -80°C until DNA extraction.

**Plasma and serum assays for one-carbon nutrients and their metabolites and biomarkers**

Detailed description, accuracy and precision of each assay have been previously reported (451, 476, 477). Serum and RBC folate concentrations were determined using quantitative, high throughput, protein-binding immunoassays, the Access Folate Assay (Beckman Coulter, Brea, CA, USA) and the Elecsys Folate Assay (Roche Diagnostics, Mannheim, Germany), respectively (451). Intra-assay and inter-assay CVs were <5% for the Access Folate Assay (serum) and <10% and <15%, respectively, for the Elecsys Folate Assay (RBC). The determined mean ± SD for serum folate using National Institute of Standards and Technology Standard Reference Material 1955 Homocysteine and Folate in Frozen Human Serum, was 4.8±0.1, 10.5±0.4, and 29.1±2.0 nmol/L for standards levels 4.5, 10, and 25 nmol/L, respectively. The CV for each level was 1.5%, 3.4%, and 6.8%, respectively. We also compared RBC folate concentrations measured by the Elecsys Folate System with those determined by microbiologic assay using chloramphenicol-resistant *Lactobacillus rhamnosus* with 5-methylTHF as the calibrator (98) in a convenience sample (n=16) of healthy male and non-pregnant female volunteers (age range: 20-63 yrs). The RBC folate values obtained with the Elecsys Folate Assay were positively correlated with those using the microbiologic assay (Pearson r=0.90, p<0.0001). On average,
RBC folate concentration values were 5.3% (40 nmol/L) higher with the Elecsys Folate Assay compared with the microbiologic assay (1171 ± 184 nmol/L versus 1131 ± 247 nmol/L) with all values within ± 20%. Plasma UMFA concentrations were determined using stable-isotope-dilution LC tandem MS as previously described (451). The limit of detection for UMFA was 0.2 nmol/L. The CV for plasma UMFA concentrations <0.9 nmol/L, 0.9-2.0 nmol/L and >2.0 nmol/L was 12.3%, 16.8%, and 4%, respectively.

Serum vitamin B₁₂ was analyzed using the Access competitive-binding immunoenzymatic assay (Beckman Coulter, Brea, CA) (476). Using the National Institute for Biological Standards and Control 03/178 WHO International Standard Vitamin B₁₂ and Serum Folate, serial dilutions of 1:1 (480 pg/ml), 1:2 (240 pg/ml) and 1:8 (60 pg/ml) concentrations were prepared and used as internal standards. Mean ± SD for each concentration were 424.1 ± 29.3 pg/ml, 163.1 ± 12.9 pg/ml, and 71.4 ± 11.4 pg/ml, respectively. The inter-assay CVs for each concentration were 6.9%, 7.9%, and 15.9%, respectively. Plasma MMA (an inverse biomarker of vitamin B₁₂ status (209)) concentrations were measured by LC-MS/MS (476). Intra and inter-assay CVs for all runs were <5%.

Plasma PLP concentrations were determined by A/C Diagnostics LLC (San Diego, CA, USA) using a non-radioactive enzymatic assay (239, 477). The inter-assay CVs for the low and high controls were 13.4 and 9.3% respectively.

Plasma total choline and betaine were measured in duplicate by LC-MS/MS (478, 479). The inter-assay coefficient of variation was ≤ 5 % for each metabolite based on duplicate measures and ≤ 6 % based on in-house controls.

Total plasma homocysteine (an inverse indicator of one-carbon nutrient status (480))
concentrations were determined by a quantitative, enzymatic assay using the Synchron LX20 System (Beckman Coulter, Brea, CA). Intra-assay and inter-assay CVs were <5.5% and <2%, respectively.

**DNA isolation and genotyping**

DNA from cord blood MNCs was isolated using the DNA Isolation Kit for Mammalian Blood (Roche Diagnostics, Mannheim, Germany). The extracted DNA was > 20 kb in all instances, was free of RNA contamination, and had an A\textsubscript{260/280} ratio between 1.8 and 2.0.

We examined the effect of 38 fetal genetic variants of 26 genes in one-carbon nutrient metabolism that are known to influence one-carbon nutrient concentrations and risk of birth outcomes and congenital defects including neural tube defects (173, 320, 481) on total 5mC and 5hmC content. The *MTHFR* 677C>T (rs1801133) and 1298A>C (rs1801131) polymorphisms were genotyped using TaqMan genotyping assays on an ABI Vii7 Biosystem (Applied Biosystems, Foster City, CA, USA) as described previously (437). The *DHFR* 19bp insertion/deletion (rs70991108) was assessed by PCR with fluorescent hybridization as previously described (332). The remaining 35 SNPs *BHMT* rs3733890; *CβS* rs234706; *CβS* rs2124459; *CβS* rs2851391; *CβS* rs4920037; *CD320* rs2336573; *CHDH* rs9001; *CHDH* rs12676; *CUBN* rs11254363; *CUBN* rs1801222; *CUBN* rs1907362; *DHFR* rs1643649; *DPEP1* rs1126464; *FUT2* rs492602; *FUT2* rs601338; *FUT2* rs602662; *GGH* rs11545076; *MMAA* rs2270655; *MTHFD1* rs2236225; *MTR* rs1805087; *MTRR* rs1801394; *MUT* rs1141321; *MUT* rs9473555; *MUT* rs4267943; *NOX4* rs11018628; *PEMT* rs7946; *PEMT* rs12325817; *PON1* rs3917577; *RFC1* rs2297291; *TCN1* rs34324219; *TCN1* rs526934; *TCN2* rs1131603; *TCN2* rs1801198; *TCN2* rs757874; *TRDMT1* rs2295809) were genotyped using the iPLEX Assay with the
MassARRAY platform (Sequenom, San Diego, CA, USA) at the Centre for Applied Genomics as described previously (438).

**Global DNA methylation and hydroxymethylation**

Global DNA methylation (5mC) and hydroxymethylation (5hmC) contents were determined using a LC-MS/MS method as previously described (482). The absolute mass of unmodified cytosine, 5mC, and 5hmC was calculated using the intensity and the known mass of the internal standards. Global DNA methylation and hydroxymethylation were measured in relation to total cytosine. Methylation of DNA was expressed as %5mC = [(5mC)/(5mC + 5hmC + unmodified C)] x 100, whereas DNA hydroxymethylation was expressed as %5hmC = [(5hmC)/(5mC + 5hmC + unmodified C)] x 100, by optimization of a previous method (483, 484).

**Statistical analysis**

Statistical analyses were performed using SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA). All available data was included in analyses and the samples size for each analysis was reported. One subject was removed because she had pernicious anemia and received vitamin B₁₂ shots. Preterm deliveries (<37 weeks gestation) were removed from the analysis (n=20; range 28-36 weeks gestation). All tests were two-tailed and an alpha level < 0.05 was considered statistically significant unless otherwise stated. Skewed variables were log transformed prior to analysis. To account for the cumulative effect of multiple methyl donors, a methyl capacity variable was created for each time point (maternal at recruitment, maternal at delivery, cord blood) representing the sum of RBC folate, serum B₁₂, plasma PLP, and plasma total choline concentrations for each time point, corrected to µmol/L. For example, methyl capacity = (nmol/L RBC folate/1000) + (pmol/L vitamin B₁₂/10^6) + (nmol/L PLP/1000) +...
µmol/L total choline. Pearson’s correlations were used to determine the association between individual one-carbon nutrients and their metabolites or biomarkers, homocysteine, and methyl capacity with 5mC and 5hmC content. All correlations were adjusted for absolute lymphocyte content (E9/L) in cord blood. DNA methylation and hydroxymethylation content was compared across quartiles of one-carbon nutrients concentrations at each time point using PROC GLM with Tukey adjustment for multiple comparisons. DNA methylation and hydroxymethylation content was compared between a subgroup of pregnant women with the highest RBC folate [Quartile 4 (Q4)] and the lowest serum B12 status [Quartile 1 (Q1) and those with more moderate RBC folate [Quartiles 1-3 (Q1-3)] and more adequate serum B12 [Quartiles 2-4 (Q2-4)] concentrations in early pregnancy and at delivery. The highest RBC folate quartile in early and late pregnancy was 2860 and 3238 nmol/L, respectively. The lowest serum B12 quartile in early and late pregnancy was 167 and 128 pmol/L, respectively. The 5mC and 5hmC content across infant genotypes was determined using PROC GLM with Tukey adjustment. Unadjusted and models adjusted for absolute lymphocyte count and maternal BMI were reported.

The Benjamini-Yekutieli procedure (dependentfdr in PROC MULTTEST) was used to account for the number of correlations investigated (12 one-carbon biomarkers with total DNA methylation and hydroxymethylation content at three time points (two for UMFA) = 71 tests) and the number of fetal genotypes (38 genetic variants with total methylation and hydroxymethylation = 76 tests) in order to maintain an overall significance level of 0.05.
7.4 Results

Characteristics of the study subjects and newborn infants

Detailed information on the PREFORM study participant characteristics has been published previously (434, 451, 476). As illustrated in Figure 7.2, 368 pregnant women enrolled in the study and 309 deliveries occurred 289 of which were full term. Figure 7.2 also details the sample size for each biomarker measured in blood which varied depending on the availability of sample. Biomarker sample sizes varied due to insufficient blood or plasma or laboratory error. The PREFORM study participants were primarily nulliparous, Caucasian, university educated pregnant women, with the mean (± SD) age of 32 ± 5 y and early pregnancy BMI of 24.6 ± 4.5 kg/m². The mean (±SD) newborn gestational age was 39.3 ± 1.7 wk. Detailed information of the newborn characteristics has been published previously (451, 476).

![Flow diagram of participant disposition and biochemical analyses at each time point](image)
**Dietary and supplemental intakes and blood concentrations of one-carbon nutrients**

Detailed information on dietary and supplemental intakes of folate/folic acid, vitamin B₁₂, vitamin B₆, choline, and betaine of the PREFORM study participants have been published previously (434). Concentrations of serum and RBC folate and plasma UMFA and homocysteine (451); plasma total choline, betaine, DMG, and TMAO (479); serum vitamin B₁₂ and plasma MMA (476); and plasma PLP (477) of the PREFORM study participants at recruitment (12-16 wk) and at delivery (37-42 wk) and of cord blood of the newborn infants have been previously published and are summarized in Table 7.1.
Table 7.1. Maternal and cord blood concentrations of one-carbon nutrients, biomarkers and homocysteine

<table>
<thead>
<tr>
<th>Nutrient/biomarker concentration</th>
<th>At recruitment (12-16 wk gestation)</th>
<th>At delivery (36-42 wk gestation)</th>
<th>Cord blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>51 (48, 54)</td>
<td>39 (37, 41)</td>
<td>64 (61, 68)</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>2416 (2361, 2472)</td>
<td>2789 (2717, 2864)</td>
<td>2681 (2607, 2758)</td>
</tr>
<tr>
<td>Plasma UMFA (nmol/L)</td>
<td>1.04 (1.03, 1.04)</td>
<td>-</td>
<td>1.03 (1.03, 1.03)</td>
</tr>
<tr>
<td>Serum B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)</td>
<td>218 (208, 227)</td>
<td>168 (161, 175)</td>
<td>320 (299, 342)</td>
</tr>
<tr>
<td>Plasma MMA (nmol/L)</td>
<td>109 (105, 114)</td>
<td>136 (129, 142)</td>
<td>313 (297, 331)</td>
</tr>
<tr>
<td>Plasma PLP (nmol/L)</td>
<td>106 (97, 116)</td>
<td>58 (54, 63)</td>
<td>296 (276, 318)</td>
</tr>
<tr>
<td>Plasma choline (µmol/L)</td>
<td>7.2 (7.0, 7.4)</td>
<td>10.8 (10.4, 11.1)</td>
<td>34.6 (33.4, 35.7)</td>
</tr>
<tr>
<td>Plasma betaine (µmol/L)</td>
<td>13.3 (12.8, 13.7)</td>
<td>10.5 (10.1, 10.8)</td>
<td>21.0 (20.5, 21.6)</td>
</tr>
<tr>
<td>Plasma DMG (µmol/L)</td>
<td>1.2 (1.2, 1.3)</td>
<td>1.4 (1.4, 1.5)</td>
<td>1.8 (1.7, 1.9)</td>
</tr>
<tr>
<td>Plasma TMAO (µmol/L)</td>
<td>1.5 (1.4, 1.6)</td>
<td>1.7 (1.6, 1.9)</td>
<td>1.5 (1.4, 1.6)</td>
</tr>
<tr>
<td>Plasma Hcy (µmol/L)</td>
<td>5.0 (4.9, 5.2)</td>
<td>6.0 (5.8, 6.2)</td>
<td>4.9 (4.7, 5.0)</td>
</tr>
</tbody>
</table>

Mean(95%CL) reported for plasma homocysteine (Hcy), choline and betaine. Geometric mean(95%CL) reported for serum folate, RBC folate, plasma unmetabolized folic acid (UMFA), serum vitamin B<sub>12</sub>, plasma pyridoxal 5’ phosphate (PLP), dimethylglycine (DMG), and trimethylamine oxide (TMAO).

**DNA methylation and hydroxymethylation of cord blood MNCs**

Mean (95% CI) content of 5mC, 5hmC, and unmodified cytosines was 5.49 % (5.44, 5.54%), 0.032% (0.030, 0.034%), and 94.48% (94.43, 94.53%), respectively. The mode of delivery (caesarian versus vaginal) or infant sexes had no effect on 5mC or 5hmC content (p>0.05).
Pregnant women with early pregnancy BMI ≥30 at baseline had significantly lower [mean (95% CI): 5.30% (5.14, 5.47%)] 5mC content in cord MNCs compared with those with BMI <30 [(5.51% (5.46, 5.57%); p=0.005]; however, there was no difference in 5hmC content (p>0.05). There were no significant associations of maternal dietary intake of folate, folic acid, vitamin B<sub>12</sub>, vitamin B<sub>6</sub>, or choline in early or mid to late pregnancy or with supplemental intakes of folic acid, vitamin B<sub>6</sub> or vitamin B<sub>12</sub> in early pregnancy with 5mC or 5hmC content in cord MNC DNA (p>0.05).

**One-carbon nutrient concentrations and 5mC in cord blood MNC DNA**

Mean (95% CI) 5mC content in cord blood MNC DNA across the quartiles of maternal and cord blood concentrations of one-carbon nutrients and their metabolites and biomarkers are presented in Appendix A. Except for maternal vitamin B<sub>12</sub> in early pregnancy and cord plasma PLP, none of the one-carbon nutrients or their metabolites and biomarkers showed any significant trend of 5mC content in cord blood MNC DNA across the quartiles of concentrations in maternal blood in early and late pregnancy or cord blood. Increasing maternal vitamin B<sub>12</sub> concentrations in early pregnancy were associated with an increasing trend of 5mC content in cord blood MNC DNA (overall p=0.01) with the third quartile of vitamin B<sub>12</sub> concentrations having 4% greater 5mC content compared with the first quartile after adjusting for multiple comparisons (p=0.02). Early pregnancy plasma DMG concentrations were weakly associated with 5mC content in cord blood MNC DNA (p=0.05) (Table A1). There were no associations with maternal concentrations at delivery and 5mC content (Table A2). Cord plasma PLP concentrations showed an increasing trend of 5mC content in cord blood MNC DNA across the quartiles (overall p=0.03) with the third quartile of cord plasma PLP concentrations showing 4.3% greater 5mC content than the first quartile after multiple comparisons adjustment (p=0.02) (Table A3).
Associations between 5mC content in cord blood MNC DNA and maternal and cord blood concentrations of one-carbon nutrients and their metabolites and biomarkers were assessed using Pearson’s correlations, adjusting for absolute lymphocyte count (Table 7.2). None of the one-carbon nutrients and their metabolites and biomarkers in maternal or cord blood except for maternal serum vitamin B$_{12}$ concentrations in early pregnancy (12-16 wk of gestation) correlated with 5mC content in cord blood MNC DNA. Maternal vitamin B$_{12}$ concentrations in early pregnancy were positively correlated with 5mC content in cord blood MNC DNA ($r=0.25$, $p=0.002$). However, it did not remain significant after adjusting for multiple testing. Using stepwise regression, a 1% increase in maternal vitamin B$_{12}$ concentrations in early pregnancy results in a 0.003% increase in 5mC content in cord blood MNC DNA ($\beta=0.003$, 95%CI [0.0002-0.006], $p=0.03$).
Table 7.2. Pearson correlations between %5mC content in cord blood mononuclear cells and one-carbon nutrients

<table>
<thead>
<tr>
<th>Nutrient/Biomarker concentration</th>
<th>At recruitment (12-16 wk gestation)</th>
<th>At delivery (37-42 wk gestation)</th>
<th>Cord blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>-0.03</td>
<td>0.09</td>
<td>-0.10</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>-0.10</td>
<td>-0.06</td>
<td>-0.04</td>
</tr>
<tr>
<td>Plasma UMFA (nmol/L)</td>
<td>0.04</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum B₁₂ (pmol/L)</td>
<td>0.25*</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>Plasma MMA (nmol/L)</td>
<td>-0.01</td>
<td>0.03</td>
<td>-0.04</td>
</tr>
<tr>
<td>Plasma PLP (nmol/L)</td>
<td>0.06</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>Plasma choline (µmol/L)</td>
<td>-0.09</td>
<td>-0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>Plasma betaine (µmol/L)</td>
<td>0.03</td>
<td>0.05</td>
<td>-0.01</td>
</tr>
<tr>
<td>Plasma DMG (µmol/L)</td>
<td>-0.03</td>
<td>0.01</td>
<td>-0.09</td>
</tr>
<tr>
<td>Plasma TMAO (µmol/L)</td>
<td>0.09</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Plasma homocysteine (µmol/L)</td>
<td>0.09</td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>Methyl capacity¹</td>
<td>-0.10</td>
<td>-0.08</td>
<td>-0.10</td>
</tr>
</tbody>
</table>

¹Serum folate, RBC folate, plasma UMFA, serum B₁₂, plasma PLP, plasma DMG and TMAO were log transformed prior to analysis. Correlations controlled for cord blood absolute lymphocyte count (E9/L).
²Methyl capacity is equal to the sum of RBC folate, serum vitamin B₁₂, plasma PLP, and free plasma choline for each subject at each time point, corrected to µmol/L.

*p=0.002, however, not significant after adjusting for multiple tests.
One-carbon nutrient concentrations and 5hmC content in cord blood MNC DNA

Mean (95% CI) 5hmC content in cord blood MNC DNA across the quartiles of maternal and cord blood concentrations of one-carbon nutrients and their metabolites/biomarkers are presented in Appendix A. There was a significant trend of 5hmC content in cord blood MNC DNA across the quartiles of the concentrations with maternal plasma PLP and TMAO in late pregnancy only (Table A2). Increasing maternal plasma PLP concentrations in late pregnancy was associated with an increasing trend of 5hmC content in cord blood MNC DNA (overall p=0.03) with the fourth quartile of plasma PLP concentrations having significantly greater 5hmC content compared with the first (p=0.05) and second (p=0.05) quartiles after adjusting for multiple comparisons. Increasing maternal plasma TMAO concentrations in late pregnancy was associated with an decreasing trend of 5mC content in cord blood MNC DNA (overall p=0.02) with the first quartile of plasma TMAO concentrations having greater 5hmC content compared with the third quartile after adjusting for multiple comparisons (p=0.02).

Associations between 5hmC content in cord blood MNC DNA and maternal and cord blood concentrations of one-carbon nutrients and their metabolites and biomarker were also assessed using Pearson’s correlations, adjusting for absolute lymphocyte count (Table 7.3). 5hmC content in cord blood MNC DNA was inversely associated with maternal RBC folate concentrations in early pregnancy (r=−0.16; p=0.04) and cord plasma UMFA concentrations (r=−0.23; p=0.004) and positively associated with plasma betaine concentrations in early pregnancy (r=0.20; p=0.01). After taking into account multiple tests, no relationships remained significant.
Table 7.3. Pearson correlations between %5hmC content in cord blood mononuclear cells and one-carbon nutrients

<table>
<thead>
<tr>
<th>Nutrient/Biomarker concentration</th>
<th>At recruitment (12-16 wk gestation)</th>
<th>At delivery (37-42 wk gestation)</th>
<th>Cord blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>-0.001</td>
<td>-0.02</td>
<td>-0.09</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>-0.16*</td>
<td>-0.03</td>
<td>-0.13</td>
</tr>
<tr>
<td>Plasma UMFA (nmol/L)</td>
<td>0.04</td>
<td>-</td>
<td>-0.23**</td>
</tr>
<tr>
<td>Serum vit. B₁₂ (pmol/L)</td>
<td>0.002</td>
<td>-0.04</td>
<td>-0.07</td>
</tr>
<tr>
<td>Plasma MMA (nmol/L)</td>
<td>0.10</td>
<td>0.05</td>
<td>-0.05</td>
</tr>
<tr>
<td>Plasma PLP (nmol/L)</td>
<td>-0.12</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>Plasma choline (µmol/L)</td>
<td>0.13</td>
<td>-0.01</td>
<td>-0.02</td>
</tr>
<tr>
<td>Plasma betaine (µmol/L)</td>
<td>0.20*</td>
<td>-0.01</td>
<td>-0.03</td>
</tr>
<tr>
<td>Plasma DMG (µmol/L)</td>
<td>-0.02</td>
<td>-0.04</td>
<td>-0.06</td>
</tr>
<tr>
<td>Plasma TMAO (µmol/L)</td>
<td>0.01</td>
<td>-0.08</td>
<td>-0.03</td>
</tr>
<tr>
<td>Plasma homocysteine (µmol/L)</td>
<td>-0.01</td>
<td>-0.06</td>
<td>-0.004</td>
</tr>
<tr>
<td>Methyl capacity²</td>
<td>0.09</td>
<td>-0.02</td>
<td>0.11</td>
</tr>
</tbody>
</table>

¹Serum folate, RBC folate, plasma UMFA, serum B₁₂, plasma PLP, plasma DMG and TMAO were log transformed prior to analysis. Correlations controlled for cord blood absolute lymphocyte count (E⁹/L).

²Methyl capacity is equal to the sum of RBC folate, serum vitamin B₁₂, plasma PLP, and plasma free choline for each subject at each time point, corrected to µmol/L.

*p<0.05, **p<0.01 however, not significant after adjusting for multiple tests
Subgroup analysis of high maternal folate and low vitamin B\textsubscript{12} concentrations on 5mC and 5hmC content in cord blood MNC DNA

5mC and 5hmC content in cord blood MNC DNA were assessed in mothers with RBC folate concentrations in the fourth quartile (Q4) and serum B\textsubscript{12} concentrations in the first quartile (Q1) compared to those with lower RBC folate (Q1-3) and higher serum B\textsubscript{12} concentrations (Q2-4). In early pregnancy, mothers with RBC folate concentrations in the fourth quartile (>2860 nmol/L) and serum B\textsubscript{12} concentrations in the first quartile (<167 pmol/L) had significantly lower 5mC content in cord blood MNC DNA [mean (95% CI); 5.16% (4.98, 5.35%)] compared with those with lower RBC folate (Q1-3) and serum B\textsubscript{12} (Q2-4) [5.50% (5.45, 5.55%)] concentrations (p=0.01). This relationship between RBC folate and serum vitamin B\textsubscript{12} concentrations was not observed for maternal blood at delivery or in cord blood.

Effect of fetal genetic variants on 5mC and 5hmC content in cord blood MNC DNA

Of the 38 fetal genetic variants investigated, significant effects on 5mC and 5hmC content in cord blood MNC DNA were observed with two genotypes: \textit{MTR} rs1805087 and \textit{GGH} rs11545076. Infants with the GG variant of the \textit{MTR} gene had significantly higher 5mC content compared to those with the AA (p=0.03) or AG genotype (p=0.05) (Figure 7.3A). The TT genetic variant of the \textit{GGH} gene had higher 5mC compared to the GT variant (p=0.003), but no difference compared to the GG variant (p=0.76) (Figure 7.3B). However, after adjusting for maternal early pregnancy BMI and cord blood absolute lymphocyte count, these findings were no longer statistically significant (p=0.07 and p=0.11 for \textit{MTR} and \textit{GGH} genes, respectively). No significant effects on 5mC or 5hmC in cord blood MNC DNA were observed with other fetal genotypes.
A.

Figure 7.3. Percent 5-methylcytosine content across fetal genotypes. A. Homozygotes for 5-methylTHF homocysteine methyltransferase (MTR) polymorphism had significantly less %5mC content than wildtype (p=0.03) and heterozygotes (p=0.05). B. Homozygotes for the gamma glutamyl hydrolase (GGH) polymorphism had significantly higher %5mC content than heterozygotes (p=0.003), but not wildtype (p=0.76). %5mC, percent 5-methylcytosine content in cord mononuclear cells.
7.5 Discussion

A new DNA methylation pattern is established during embryogenesis soon after implantation (381) and alterations in this dynamic process have been proposed to be one of the underpinning mechanisms for the development of disease in the offspring later in life (353). DNA methylation of the developing fetus appears to be highly susceptible to maternal nutrition, in particular one-carbon nutrients that are integrally related to the generation of SAM, the universal methyl donor for most of biological methylation reactions, including DNA methylation (395). Although maternal supplementation of the combination of one-carbon nutrients including folic acid, vitamin B12, choline and betaine or of folic acid alone during pregnancy has been shown to influence DNA methylation of the offspring with consequent phenotypic changes and disease susceptibility in animal models (79, 80, 397, 405, 406, 468), only limited support for this effect exists in human studies (414-420, 423, 424). A small study from the United Kingdom reported an inverse correlation between cord plasma homocysteine concentrations and LINE-1 DNA methylation in cord blood lymphocytes, although no significant correlations were observed for cord serum folate concentrations (414). Cross-sectional examination of a population unexposed to folic acid supplements before and during pregnancy revealed no significant associations between promoter DNA methylation of the IGF2 gene in cord blood lymphocytes and serum folate concentrations in either mother’s or umbilical cord blood (416). However, in an observational study from The Netherlands, periconceptional maternal folic acid use of 400 µg/day significantly increased, by 4.5 %, DNA methylation of the IGF2 DMR in whole blood obtained from children at 17 months of age (417). More recently, the NEST Study reported that periconceptional folic acid use had no effect on DNA methylation at the IGF2 DMR but significantly decreased DNA methylation at the H19 DMR in cord blood leukocytes compared to
non-users (418). Boeke et al. did not find a significant relationship between LINE-1 leukocyte DNA methylation and dietary methyl intakes, including folate, vitamin B$_{12}$, choline and betaine in a folate-replete population (424).

In the present study, we determined the association of maternal and cord blood concentrations of one-carbon nutrients and their metabolites and biomarkers with the global content of 5mC and 5hmC in cord blood MNC DNA in a cohort of pregnant Canadian women and their newborns. Our a priori hypothesis was that maternal one-carbon nutrient concentrations would positively correlated with 5mC content in cord blood MNC DNA, given one-carbon nutrients’ role in the generation of SAM (1). Furthermore, we posited that this correlation would be more pronounced in maternal one-carbon nutrient concentrations in early pregnancy, given that DNA methylation is programmed during early embryogenesis (353). Contrary to our expectation, however, none of maternal one-carbon nutrients and their metabolites and biomarkers in early pregnancy except for serum B$_{12}$ concentrations, which were positively, albeit modestly, correlated with 5mC content in cord blood MNC DNA. Vitamin B$_{12}$ functions as the coenzyme of methionine synthase in the remethylation of homocysteine to methionine, thereby playing a critical role in the generation of SAM (1). As expected, none of maternal one-carbon nutrient concentrations in late pregnancy was associated with 5mC in cord blood MNC DNA as newly established DNA methylation in the fetus is unlikely influenced by maternal blood concentrations of one-carbon nutrients in late pregnancy. Cord blood concentrations of one-carbon nutrients and their metabolites and biomarkers, except for plasma PLP, had largely no effect on 5mC content of cord blood MNC DNA, likely because DNA methylation pattern of the newborn infant had already been established in utero.

The general lack of association of maternal concentrations of one-carbon nutrients and their
metabolites and biomarkers in early pregnancy with 5mC content in cord blood MNC DNA is surprising. However, several plausible explanations exist to account for the lack of effect and association. As previously reported, the PREFORM study participants’ geometric mean (95% CI) RBC folate concentration was 2417 nmol/L (2362, 2472 nmol/L) (451). This RBC folate concentration is in the range generally considered to be very high. As comparison, RBC folate concentration corresponding to the 97th percentile of the NHANES (1999-2004) data is 1360 nmol/L. This observed high RBC folate concentrations in our study participants are likely related to the high proportion of women who consumed folic acid supplements during the first trimester (93%) and the high dose of folic acid (mean daily dose of 1000 µg) (434). Therefore, it is possible that the observed high maternal RBC folate concentrations in early pregnancy, and in fact throughout the entire pregnancy, might have masked any effect of folate/folic acid and other one-carbon nutrients on 5mC content in cord blood MNC DNA. We have arbitrarily developed total methyl capacity score, which is equal to the sum of RBC folate, serum vitamin B12, plasma PLP, and total choline, to determine the combined effect of the four major one-carbon nutrients on 5mC content in cord blood MNC DNA. Again, we did not observe any significant association of total methyl capacity score with 5mC content in cord blood MNC DNA.

The effect of maternal status of one-carbon nutrients on 5hmC in cord blood MNC DNA has not previously been examined. We observed an inverse association of maternal RBC folate concentrations, whereas a positive association of maternal betaine concentrations, in early pregnancy with 5hmC content in cord blood MNC DNA. However, maternal concentrations of one-carbon nutrients and their metabolites and biomarkers in late pregnancy had no effect on 5hmC. Cord plasma UMFA concentrations were inversely associated with 5hmC content of cord blood MNC DNA. Hydroxymethylation of DNA is a recently described epigenetic marker of the
mammalian genome generated by the oxidation of 5mC (366). The conversion of 5mC to 5hmC is an active process by ten-eleven-translocation proteins and is independent of one-carbon metabolism (358, 366). 5hmC is considered an intermediate product of the demethylation process (358) and a fundamental epigenetic marker related to the process of cellular differentiation and carcinogenesis (485). At present, however, the exact biological function and its role in human health and disease have not been clearly established.

Due to the large number of tests (n=71 correlations, n=76 genotype associations), it is important to note we would expect approximately 4 significant associations to arise purely by chance. Therefore, after adjusting for the number of tests, none of the aforementioned significant associations remained significant. Our sample size was relatively small and likely did not have sufficient power to obviate this limitation. Furthermore, all of the correlations were considered negligible to weak with all |r| values < 0.26. Notwithstanding this limitation, our observation that maternal concentrations of few individual one-carbon nutrients in early pregnancy may influence DNA methylation and hydroxymethylation in the developing fetus is novel and warrants confirmation and elucidation of functional ramifications.

One interesting finding was the decreased global DNA methylation in cord blood MNCs in the mothers with blood concentrations of high folate/low vitamin B₁₂ in early pregnancy. This combination of high folate and low vitamin B₁₂ status in mothers during pregnancy has previously been related to an increased risk for small-for-gestational-age babies (6) and childhood adiposity and insulin resistance at age six (475). The observed decrease in DNA methylation resulting from high folate/low vitamin B₁₂ status could be explained by the methyl trap hypothesis and other mechanisms. Vitamin B₁₂ functions as the coenzyme of MS, which catalyzes the transfer of a methyl group from 5-methylTHF to remethylate homocysteine to
methionine (46). In the setting of inadequate levels of vitamin B₁₂, methionine synthase activity is reduced and therefore, folate is “trapped” as 5-methylTHF (46). This results in lower levels of SAM and consequent lower DNA methylation. The methyl trap is usually accompanied by an increase in homocysteine. However, we did not observe elevated plasma homocysteine concentrations in this subgroup [mean (95% CI): 4.8 (4.2, 5.5 µmol/L) and 5.0 (4.8, 5.1 µmol/L) in Q4 RBC folate/Q1 B₁₂ mothers and Q1-Q3 RBC folate/Q4 serum B₁₂ mothers, respectively, in early pregnancy]. This is likely due to adequate levels of choline and betaine, which ensures the remethylation of homocysteine to methionine via BHMT and/or of vitamin B₆, which metabolizes homocysteine to cystathionine via CβS. Other explanations for this finding could be through the inhibitory action of folate and folic acid on MTHFR via increased SAM (368) or DHF (486) concentrations. MTHFR inhibition would lower 5-methylTHF levels, further exacerbating the remethylation of homocysteine to methionine by MS (367), which is already inhibited by low vitamin B₁₂ status.

Our exploratory analysis suggests that two fetal genetic variants in the one-carbon metabolic pathways may influence DNA methylation in cord blood MNCs. Of note, we found infants with the GG variant for the MTR gene, which encodes methionine synthase, had significantly higher 5mC content compared to those with the AA or AG genotype. This MTR genotype is associated with increased methionine synthase activity as indicated by lower plasma homocysteine concentrations (323, 487-489), resulting in higher methionine and consequently SAM concentrations for DNA methylation. The GG variant has previously been shown to be associated with lower homocysteine concentrations and higher DNA methylation in adults (490), although not all studies have found this relationship (491).
The PREFORM study is a large comprehensive observational study in a demographically diverse group of pregnant North American women and their infants. We comprehensively examined the association between concentrations of four major one-carbon nutrients and their metabolites and biomarkers in both early and late pregnancy maternal blood and in cord blood on total DNA methylation and hydroxymethylation. Furthermore, this is the first study that examined the association of maternal and cord blood one-carbon nutrient status on DNA hydroxymethylation in the newborn infant. However, there are several limitations associated with our study. We measured DNA methylation and hydroxymethylation in cord blood MNCs. As DNA methylation and hydroxymethylation are cell and tissue-specific, the observed changes in these epigenetic markers observed in MNCs do not reflect those in other cells or tissues. Furthermore, we used total MNCs to measure DNA methylation and hydroxymethylation. As MNCs are consisted of several types of cells, we should have extracted a specific MNC type to analyze DNA methylation and hydroxymethylation. As lymphocytes make up the majority of MNCs, however, we controlled for the absolute lymphocyte count in cord blood when analyzing DNA methylation and hydroxymethylation. We did not determine site and gene-specific DNA methylation in the present study and studies are currently underway to investigate the effect of maternal and cord blood one-carbon nutrient concentrations on gene-specific DNA methylation using an epigenomic approach and its functional effect on gene expression.

Although the magnitude of effect was modest, our data suggest that maternal concentrations of some individual one-carbon nutrients in early pregnancy may influence DNA methylation and hydroxymethylation in cord blood MNCs. However, given the large number of tests, we cannot rule out chance findings. Future studies are warranted to confirm our findings in a larger sample
size and elucidate functional ramifications of the observed effect and its impact on health and disease susceptibility of the offspring in a follow-up study.
Chapter 8: Overall Conclusions

8.1. Overall Discussion

The primary objective of this thesis was to determine whether or not maternal status of one-carbon nutrients and their metabolites and biomarkers affect DNA methylation and hydroxymethylation in the newborn infant. It was hypothesized maternal one-carbon nutrients would have a positive association with DNA methylation and an association with DNA hydroxymethylation. We determined and characterized the folate and vitamin B$_6$ status of a cohort of Canadian pregnant women during early and late pregnancy and in cord blood. In the first study described in Chapter 5, maternal and cord concentrations of serum and RBC folate were generally considered high, largely due to ubiquitous folic acid supplementation before and during pregnancy (Figure 8.1). Furthermore, detectable levels of UMFA were found in the majority of maternal plasma samples in early pregnancy and in cord plasma. In the second study described in Chapter 6, vitamin B$_6$ status, as assessed by plasma PLP concentrations, were largely adequate ($\geq$20 nmol/L) in maternal plasma in early and late pregnancy. Cord plasma PLP concentrations were found to be significantly higher and were associated with maternal status. Fetal genetic variants had little effect on cord concentrations of folate, PLP, and homocysteine in both studies. Finally, in the third study described in Chapter 7, few individual maternal one-carbon nutrients were associated with DNA methylation and hydroxymethylation in cord blood MNCs. However, due to the exploratory nature of the analysis, no associations remained significant after adjusting for multiple tests. Overall, this research provides a framework for future studies investigating optimal folate and vitamin B$_6$ for pregnant women and for the developing fetus and a basis for future analyses aimed at elucidating the effects of maternal one-
carbon nutrients on DNA methylation and hydroxymethylation in the newborn infant and their functional ramifications.

**Figure 8.1: Summary of thesis conclusions**

One-carbon nutrients (folate, vitamins B₆ and B₁₂, choline and betaine) play a critical role in fetal and early life development. Increased maternal consumption and improved status of these nutrients have been shown to reduce, albeit equivocally except for NTDS for folate, the risk of adverse pregnancy and birth outcomes and may improve health outcomes and modify disease susceptibility of the offspring. However, excessive intakes, particularly folic acid intake, are increasingly prevalent and their consequences need to be addressed. Although maternal folate status has been previously characterized, little data exist on the folate status of North American
pregnant women exposed to mandatory folic acid fortification and widespread use of prenatal folic acid supplementation. Our study is the largest in North America to include a comprehensive analysis of both maternal and cord blood folate biomarkers, including novel plasma UMFA concentrations. Our findings confirm that the developing fetuses are exposed to high folate and folic acid concentrations during the intrauterine period due to high folate and folic acid intake and blood concentrations in the mothers. Additionally, our study is the largest to characterize PLP concentrations in maternal and cord plasma. Recently, concerns have been raised regarding inadequate vitamin B6 intakes in the North American population. However, our findings suggest maternal vitamin B6 status is adequate for the majority of the population, most likely due to widespread supplementation during pregnancy. Finally, our study is the largest and most comprehensive in addressing whether four major one-carbon nutrients, individually and collectively, affect global DNA methylation and the novel DNA hydroxymethylation in cord blood MNCs. Although only modest associations were observed in our exploratory analysis, our findings provide evidence that maternal status of one-carbon nutrients, particularly in early pregnancy, is associated with DNA methylation and hydroxymethylation. As even a small magnitude of change in DNA methylation may be associated a significant functional ramification, further studies are warranted to elucidate the precise nature and its functional ramifications of one-carbon nutrient-associated DNA methylation and hydroxymethylation alterations during pregnancy.

8.2. Strengths and Limitations

To date, the PREFORM Study is the largest Canadian cohort study that investigated how one-carbon nutrient status affects global DNA methylation and hydroxymethylation in the offspring.
Pregnant women are often excluded from or underrepresented in large national surveys. Therefore, our data from a cohort of pregnant Canadian women provide important information that is often not gathered in large national surveys. While our cohort is highly reflective of the multiethnic population of Toronto, our findings may not represent all Canadians. Nonetheless, our data significantly adds to the growing body of evidence that suggests that maternal nutrition may have lasting epigenetic effects in the offspring. Also, we are one of the few studies, which measured global DNA methylation and hydroxymethylation using the sophisticated LC/MS-MS method. Other DNA methylation analyses, most of which utilizes bisulfite treatment, do not distinguish between a methylated or hydroxymethylated cytosine, while we were able to precisely quantify each. Emerging evidence suggests 5mC and 5hmC have different and in certain cases, opposing effects on gene expression and function, and hence, distinguishing between the two epigenetic modifications is important. Another strength of our study is the broad spectrum of nutrients assessed. We interrogated the effect of all 4 major one-carbon nutrients and their metabolites and biomarkers, individually and collectively, on DNA methylation and hydroxymethylation. We also established a biorepository. We have amassed a large volume of plasma aliquots from maternal and cord blood and isolated MNCs in cord blood, which can be used for future analyses using DNA, RNA, and protein.

There are a few limitations to this study. Although FFQs are simple tools to assess diet in larger populations, they are limited by their semi-quantitative data, making it difficult to assess true dietary deficiencies or excesses which are better evaluated with other quantitative assessment tools (24 hour recalls/3 day food record). We did not collect fasting blood samples from our subjects. Fasting status has been shown to affect concentrations of nutrients, namely serum

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Folate and PLP. However, we recruited study participants at the first obstetrical appointment when blood was drawn therefore it was not possible to prepare the participant to fast. Furthermore, sensitivity analyses did not show any differences in serum folate or plasma PLP concentrations between samples taken < 5 hours or ≥ 5 hours from the time of last meal or supplement intake. Although we comprehensively genotyped for 39 SNPs in the offspring, we did not assess genotyping information from mothers. Maternal genotype data would have strengthened our understanding of factors that influence maternal and cord blood concentrations of one-carbon nutrients, including investigating interactions between maternal and fetal genotypes. We also did not separate lymphocytes from other MNCs when assessing 5mC and 5hmC content. As DNA methylation and hydroxymethylation are cell and tissue-specific, future studies should ensure DNA methylation and hydroxymethylation analyses are performed in specific cell type and avoid contamination with other cell types. Furthermore, we did not assess potential functional outcomes of DNA methylation and hydroxymethylation changes associated with early pregnancy high RBC folate and low serum B₁₂ status and we did not interrogate gene-specific methylation and hydroxymethylation. Although we powered this study to detect a correlation between serum folate and DNA methylation, a larger sample size would have been needed to detect an association between other one-carbon nutrients; however, we can use our exploratory analysis to power future studies to identify relationships between a specific one-carbon nutrient and DNA methylation. Finally, we could not correlate maternal and cord blood one-carbon nutrient concentrations with pregnancy and/or birth outcomes in our study as these adverse outcomes were very small.
8.3. Future Directions

Our data provide a framework for future studies aimed at elucidating the relationship between maternal one-carbon nutrient status and DNA methylation and hydroxymethylation in the human offspring and their functional ramifications and long-term health consequences. Some potential future studies include: 1) design of a similar study in an animal model, potentially an outbred strain of guinea pig given its similarities to human placental transfer and delivery of well developed offspring (411), to address the primary objective of the PREFORM Study; 2) collection of nation-wide data to better capture the nutrient status of all Canadian women and thus, enable the identification of high risk subgroups that are susceptible to deficiency or excess of one-carbon nutrients during pregnancy; 3) design and implement follow up studies in the offspring to investigate the lasting effect of maternal one-carbon nutrient status on DNA methylation and hydroxymethylation, anthropometric and metabolic outcomes, and health outcomes. In particular, investigating body composition and metabolic outcomes in the adolescent offspring from the mothers with high folate and low vitamin B\textsubscript{12} in early pregnancy may be very interesting, considering previous evidence has suggested higher risk of insulin resistance and obesity in these children (8); 4) determination of gene-specific DNA methylation and hydroxymethylation in cord blood MNCs associated with maternal one-carbon nutrient status and their functional ramifications; 5) conduct a similar study in non-fortified countries to assess association of one-carbon nutrients on DNA methylation and hydroxymethylation in the newborn infants exposed to lower folate levels in utero and potentially elucidate the relationship between folate and DNA methylation not seen in our population.
8.4. Final Conclusions

Overall, this thesis determined maternal and cord blood concentrations of folate were higher than previously reported values in other pregnant populations, while plasma PLP concentrations were generally adequate. For both nutrients, prenatal supplement use was associated with higher nutrient concentrations and maternal concentrations were associated with cord concentrations. Fetal genetic polymorphisms of one-carbon metabolism influenced cord folate concentrations, but not PLP concentrations. Finally, maternal concentrations of folate, vitamin B\textsubscript{12}, and betaine as well as those with a combination of high folate and low B\textsubscript{12} in early pregnancy appeared to exert the most influence on DNA methylation in cord MNCs. Collectively, these findings suggest public health recommendations of the use of prenatal supplements before and during pregnancy may be effective considering the majority of participants reported use. However, the recommended dose of vitamins, particularly folic acid, does not always match what is commonly marketed to North American consumers. This was apparent by supplemental folic acid intakes above the recommendation and consequent high blood folate concentrations in our population. Finally, the fact that we did not find a strong association between maternal one-carbon nutrient status and fetal epigenetic effects does not indicate this relationship does not exist. There are many other factors beyond maternal one-carbon nutrient status such as stress, chemical exposures, as well as postnatal exposures that play a role in modulating DNA methylation in the offspring.

Overall, our findings are reassuring for adequate maternal vitamin B\textsubscript{6} status for the majority of women but support the need to address whether or not high folate exposure is detrimental to either maternal or fetal health. Furthermore, the exploratory analysis on the effects of one-
carbon nutrients on DNA methylation and hydroxymethylation in the offspring provide a framework for future studies. Collectively, data from the PREFORM Study will contribute to the body of evidence on maternal nutrient status to help shape public health guidelines and policies about one-carbon nutrient exposure during pregnancy for optimal pregnancy and birth outcomes and offspring’s health. Future studies are warranted to determine how the nutritional milieu \textit{in utero} can have a lasting impact on the health of the offspring.
References


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## Appendix A

Table 1A: Mean (95% CI) 5mC and 5hmC percent content across quartiles of early pregnancy (12-16 weeks gestation) maternal blood of one-carbon nutrients and their metabolites/biomarkers and methyl capacity

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>%5mC content</th>
<th>%5hmC content</th>
<th>Overall p-value</th>
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<td>Q1 (nmol/L)</td>
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<td>Serum folate</td>
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<tr>
<td>Plasma DMG (µmol/L)</td>
<td>Q1 5.45 (5.35, 5.55)</td>
<td>Q2 5.61 (5.51, 5.71)</td>
<td>Q3 5.41 (5.31, 5.52)</td>
<td>Q4 5.48 (5.38, 5.58)</td>
</tr>
<tr>
<td>Plasma TMAO (µmol/L)</td>
<td>Q1 5.46 (5.36, 5.56)</td>
<td>Q2 5.47 (5.37, 5.58)</td>
<td>Q3 5.51 (5.41, 5.61)</td>
<td>Q4 5.50 (5.40, 5.60)</td>
</tr>
<tr>
<td>Plasma Hcy (µmol/L)</td>
<td>Q1 5.46 (5.38, 5.55)</td>
<td>Q2 5.44 (5.35, 5.53)</td>
<td>Q3 5.59 (5.48, 5.69)</td>
<td>Q4 5.49 (5.30, 5.68)</td>
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<tr>
<td>Methyl capacity</td>
<td>Q1 5.45 (5.35, 5.55)</td>
<td>Q2 5.58 (5.48, 5.68)</td>
<td>Q3 5.49 (5.38, 5.59)</td>
<td>Q4 5.44 (5.33, 5.55)</td>
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</table>
Table 2A: Mean (95% CI) 5mC and 5hmC percent content across quartiles of maternal blood concentrations of one-carbon nutrients and their metabolites/biomarkers and methyl capacity at the time of delivery

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<tr>
<th>Biomarker</th>
<th>%5mC content</th>
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<th>Overall p-value</th>
<th>%5mC content</th>
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<th>Overall p-value</th>
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<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>Q1</td>
<td>5.44 (5.34, 5.55)</td>
<td>5.53 (5.42, 5.64)</td>
<td>5.51 (5.41, 5.62)</td>
<td>5.50 (5.39, 5.60)</td>
<td>0.69</td>
<td>0.031 (0.027, 0.035)</td>
<td>0.031 (0.027, 0.035)</td>
<td>0.033 (0.030, 0.037)</td>
<td>0.030 (0.026, 0.034)</td>
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<tr>
<td>RBC folate (nmol/L)</td>
<td>Q2</td>
<td>5.55 (5.45, 5.65)</td>
<td>5.53 (5.43, 5.64)</td>
<td>5.46 (5.35, 5.56)</td>
<td>5.42 (5.31, 5.53)</td>
<td>0.26</td>
<td>0.032 (0.028, 0.036)</td>
<td>0.033 (0.030, 0.037)</td>
<td>0.031 (0.027, 0.035)</td>
<td>0.030 (0.026, 0.034)</td>
</tr>
<tr>
<td>Serum B12 (pmol/L)</td>
<td>Q3</td>
<td>5.41 (5.31, 5.51)</td>
<td>5.55 (5.45, 5.64)</td>
<td>5.52 (5.42, 5.62)</td>
<td>5.48 (5.37, 5.58)</td>
<td>0.26</td>
<td>0.033 (0.029, 0.037)</td>
<td>0.030 (0.026, 0.034)</td>
<td>0.032 (0.028, 0.035)</td>
<td>0.031 (0.027, 0.035)</td>
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<tr>
<td>Plasma MMA (nmol/L)</td>
<td>Q4</td>
<td>5.43 (5.33, 5.53)</td>
<td>5.58 (5.48, 5.69)</td>
<td>5.46 (5.35, 5.56)</td>
<td>5.52 (5.41, 5.62)</td>
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<td>0.031 (0.027, 0.035)</td>
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<tr>
<td>Plasma PLP (nmol/L)</td>
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<td>5.49 (5.38, 5.66)</td>
<td>5.58 (5.47, 5.68)</td>
<td>5.52 (5.41, 5.62)</td>
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<td>0.029 (0.025, 0.033)</td>
<td>0.032 (0.028, 0.036)</td>
<td>0.036 (0.032, 0.040)</td>
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<tr>
<td>Plasma choline (µmol/L)</td>
<td>Q2</td>
<td>5.53 (5.42, 5.63)</td>
<td>5.48 (5.37, 5.58)</td>
<td>5.50 (5.40, 5.60)</td>
<td>5.47 (5.36, 5.57)</td>
<td>0.86</td>
<td>0.032 (0.028, 0.036)</td>
<td>0.033 (0.029, 0.037)</td>
<td>0.033 (0.030, 0.037)</td>
<td>0.028 (0.024, 0.032)</td>
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<tr>
<td>Plasma betaine (µmol/L)</td>
<td>Q3</td>
<td>5.52 (5.42, 5.63)</td>
<td>5.45 (5.35, 5.63)</td>
<td>5.47 (5.37, 5.57)</td>
<td>5.53 (5.42, 5.63)</td>
<td>0.69</td>
<td>0.033 (0.029, 0.037)</td>
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<td>0.034 (0.030, 0.037)</td>
<td>0.031 (0.027, 0.035)</td>
</tr>
<tr>
<td>Plasma DMG (µmol/L)</td>
<td>Q4</td>
<td>5.47 (5.36, 5.57)</td>
<td>5.51 (5.41, 5.62)</td>
<td>5.49 (5.38, 5.59)</td>
<td>5.50 (5.40, 5.60)</td>
<td>0.96</td>
<td>0.030 (0.026, 0.034)</td>
<td>0.035 (0.031, 0.039)</td>
<td>0.031 (0.028, 0.035)</td>
<td>0.030 (0.026, 0.034)</td>
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<tr>
<td>Plasma TMAO (µmol/L)</td>
<td>Q1</td>
<td>5.46 (5.36, 5.56)</td>
<td>5.47 (5.36, 5.57)</td>
<td>5.48 (5.38, 5.58)</td>
<td>5.56 (5.46, 5.67)</td>
<td>0.46</td>
<td>0.034 (0.031, 0.038)</td>
<td>0.033 (0.029, 0.037)</td>
<td>0.026 (0.023, 0.030)</td>
<td>0.032 (0.028, 0.036)</td>
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<tr>
<td>Plasma homocysteine (µmol/L)</td>
<td>Q2</td>
<td>5.49 (5.41, 5.57)</td>
<td>5.45 (5.35, 5.56)</td>
<td>5.48 (5.34, 5.61)</td>
<td>5.55 (5.42, 5.67)</td>
<td>0.71</td>
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<td>0.035 (0.032, 0.039)</td>
<td>0.029 (0.025, 0.034)</td>
<td>0.029 (0.024, 0.033)</td>
</tr>
<tr>
<td>Methyl capacity</td>
<td>Q3</td>
<td>5.47 (5.37, 5.58)</td>
<td>5.54 (5.44, 5.64)</td>
<td>5.53 (5.43, 5.64)</td>
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<td>0.41</td>
<td>0.031 (0.027, 0.035)</td>
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<td>0.032 (0.028, 0.036)</td>
<td>0.028 (0.024, 0.032)</td>
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</table>
Table 3A. Mean (95% CI) 5mC and 5hmC percent content across quartiles of cord blood concentrations of one-carbon nutrients and their metabolites/biomarkers and methyl capacity

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<thead>
<tr>
<th>Biomarker</th>
<th>%5mC content</th>
<th>%5hmC content</th>
<th>Overall p-value</th>
<th>%5mC content</th>
<th>%5hmC content</th>
<th>Overall p-value</th>
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<tbody>
<tr>
<td>Serum folate (nmol/L)</td>
<td>5.55 (5.44, 5.66)</td>
<td>5.51 (5.40, 5.63)</td>
<td>5.52 (5.41, 5.64)</td>
<td>5.42 (5.31, 5.53)</td>
<td>0.41</td>
<td>0.031 (0.026, 0.035)</td>
</tr>
<tr>
<td>RBC folate (nmol/L)</td>
<td>5.54 (5.43, 5.65)</td>
<td>5.47 (5.36, 5.57)</td>
<td>5.46 (5.35, 5.56)</td>
<td>5.47 (5.36, 5.58)</td>
<td>0.68</td>
<td>0.035 (0.031, 0.039)</td>
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<td>Plasma UMFA (nmol/L)</td>
<td>5.44 (5.32, 5.56)</td>
<td>5.51 (5.40, 5.63)</td>
<td>5.58 (5.46, 5.70)</td>
<td>5.47 (5.36, 5.59)</td>
<td>0.40</td>
<td>0.032 (0.028, 0.037)</td>
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<tr>
<td>Serum B₁₂ (pmol/L)</td>
<td>5.40 (5.29, 5.51)</td>
<td>5.53 (5.41, 5.64)</td>
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<td>Plasma MMA (nmol/L)</td>
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<td>0.031 (0.027, 0.035)</td>
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<td>Plasma betaine (µmol/L)</td>
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<td>Q2</td>
<td>Q3</td>
<td>Q4</td>
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