The Effect of Maternal Vitamin B_{12} Status in Combination with High Folate Status on Gene-Specific DNA Methylation in Cord Blood Mononuclear Cells

by:

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

Department of Nutritional Sciences
University of Toronto

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Abstract

DNA methylation is an important epigenetic determinant in gene expression, with potential consequent effects on disease susceptibility. Maternal folate and B\textsubscript{12} has the potential to modulate DNA methylation via the provision of S-adenosylmethionine. Using the Illumina Infinium MethylationEPIC array, we found lower genome-wide (-0.0059±0.0147, p=0.013) and gene-specific (p=0.007) DNA methylation in genes influenced by maternal low serum B\textsubscript{12} and high RBC folate concentrations at early pregnancy. Analyses using Ingenuity Pathway Analysis and ddPCR determined that B\textsubscript{12} and folate-induced DNA methylation changes altered expression of functionally relevant genes related to metabolism and development. Infants born to mothers with a low B\textsubscript{12} status are shown to have larger infant anthropometric measures. Our data suggest that low B\textsubscript{12} and high folate induced DNA methylation changes in the developing fetus. Given the potential for these changes to mediate disease risk later in life, future large-scale studies are warranted to confirm our exploratory findings.
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“The noblest pleasure is the joy of understanding.” Although I do not have a double-blinded, placebo-controlled, randomized control trial to support Leonardo da Vinci’s statement, what I do have are an abundance of experiences that formed this unforgettable journey of learning that is my Master’s program. I have gained invaluable lessons and have grown my understanding of scientific research and things concerning life. This Master’s has granted me the opportunity to be curious, which has allowed me to produce this work. I am truly blessed to have been part of a Master’s program at the University of Toronto’s Department of Nutritional Sciences.

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Abbreviations

5hmC, 5-hydroxymethylation
5mC, 5-methylcytosine
5-MTHF, 5-methyltetrahydrofolate
Ai, Adequate Intake
APrON, Alberta Pregnancy Outcomes in Nutrition
ChIP, chromatin immunoprecipitation
CpG, cytosine-phosphate-guanine dinucleotide
CRC, colorectal cancer
ddPCR, digital droplet polymerase chain reaction
DFE, dietary folate equivalent
DHF, dihydrofolate
DHFR, dihydrofolate reductase
DMR, differentially methylated region
DNMT, DNA methyltransferases
DRI, dietary reference intake
DOHaD, Developmental Origins of Health and Disease
dUMP, deoxyuridine-5-monophosphate
dUTP, deoxythmidine-5-monophosphate
EAR, Estimated Average Requirement
FFQ, food frequency questionnaire
Hcy, homocysteine
HDAC, histone deacetylase
HoloTC, holotranscobalamin
IUGR, intrauterine growth retardation
LINE-1, long interspersed nucleotide element-1
MMA, methylmalonic acid
MNC, mononuclear cell
MS/MTR, methionine synthase / methyltransferase
MSR/MTRR, methionine synthase reductase
MTHFR, methylenetetrahydrofolate reductase
NHANES, National Health and Nutrition Examination Survey
NTD, neural tube defect
PREFORM, PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant
RBC, red blood cell
RDA, Recommended Dietary Allowance
SAH, S-adenosylhomocysteine
SAM, S-adenosylmethionine
THF, tetrahydrofolate
TS, thymidylate synthase
UMFA, unmetabolized folic acid
UL, tolerable upper limit
WCBA, women of childbearing age
1- Introduction

Folate and B₁₂ play an important role in one-carbon metabolism which is involved in a variety of biological methylation reactions including DNA methylation¹. DNA methylation of cytosine-guanine (CpG) sites, which can occur in clusters called ‘CpG islands’ located within the promoter regions of genes, inversely, albeit with a few exceptions, often regulates gene transcription and expression. In contrast, CpG methylation within the gene body also ensures genomic stability⁴. Aberrant patterns and dysregulation of DNA methylation are mechanistically related to the pathogenesis of several human diseases including cancer⁴. In vitro animal as well as human studies have shown that folate and B₁₂ status can modulate DNA methylation in a cell or tissue, and in a site and/or gene-specific manner through its effects on S-adenosylmethionine (SAM), the universal methyl donor. However, results from these studies have not been uniformly consistent⁵. DNA methylation is reprogrammed during the embryogenesis and is maintained postnatally⁶. Metabolic and hormonal in utero environment has been shown to critically affect DNA methylation programming of the fetus with consequent effects on disease susceptibility and health in adulthood⁷.

Maternal diet during pregnancy has garnered significant attention due to its potential to modulate DNA methylation⁴,⁸. Maternal status of folate and B₁₂, either individually or in combination, have been shown to be important in fetal development and growth. Suboptimal maternal status of these B vitamins during pregnancy has been associated with adverse pregnancy and birth outcomes and disease susceptibility of the offspring. Although optimal maternal folate and B₁₂ status have been shown to be protective for certain birth and pregnancy outcomes, excess folate and/or B₁₂ status has been shown to be associated with adverse health outcomes in the offspring⁹.
The imbalance of folate and $B_{12}$, that is a high folate/low $B_{12}$ status, has been associated with more pronounced adverse outcomes in the offspring. Since the levels of folate and $B_{12}$ vary between studies, we have set $\geq 1360$ nmol/L as high RBC folate concentration and $\leq 150$ pmol/L as a low $B_{12}$ serum concentration. The high folate cutoff is based on the 97$^{th}$ percentile of RBC folate concentrations using the 1999-2004 NHANES data, and the low $B_{12}$ cutoff is a commonly considered a deficiency cutoff. Two prospective observational studies in India have reported that high folate and low $B_{12}$ intakes during pregnancy are associated with small for gestational age babies and blood concentrations with insulin resistance and adiposity in the offspring at six years of age. However, mechanisms by which high folate and low $B_{12}$ status during pregnancy affect infant health outcomes have not been elucidated yet. We posit that high folate and low $B_{12}$ status during pregnancy modulates DNA methylation of critical genes involved in growth and development and predispose the offspring to metabolic syndrome and obesity.

Hence, my overall research question is: can maternal folate and $B_{12}$ status have an epigenetic effect in the offspring, which may lead to functional, biological and clinical ramifications related to fetal growth and development? I hypothesize that maternal folate and $B_{12}$ nutrient exposure will directly affect DNA methylation in genes associated with fetal growth and development.
2- Literature Review

2.1 Folate/Folic Acid

2.1.1 Definition, Chemical Structure and Dietary Sources

Folate is a water-soluble B vitamin (B<sub>9</sub>) in its naturally occurring form, while folic acid is synthetically manufactured<sup>5</sup>. Folate encompasses naturally occurring dietary folates and all biochemically related compounds including folic acid<sup>14</sup>. The common chemical structure of folate and folic acid are the 2-amino-4-hydroxy-pteridine moiety (pterin ring) attached to p-aminobenzoic acid (PABA) by a methylene bridge and glutamate residues attached by \( \gamma \)-peptide bonds (Figure 2.1.1). Dietary folates (pteroylpolyglutamates) are characterized as having polyglutamylated tails and reduced pterin ring, while folic acid (pteroylmonoglutamic acid) contain monoglutamylated tails and an oxidized ring (Figure 2.1.1)<sup>5</sup>.

**Figure 2.1.1.** Chemical structure of folate (a) and folic acid (b). The common features entail a pterin (pteridine) ring attached to PABA by a methylene bridge with glutamate residues attached by \( \gamma \)-peptide bonds. The pterin ring of dietary folate are reduced and contain more than one glutamate residue (polyglutamylated), while in folic acid the pterin ring is fully oxidized at the N5 and N10 positions and contain one glutamate residue (monoglutamylated). Reprinted with permission from Warzyszynska 2014<sup>5</sup>. 
The main naturally occurring sources of dietary folate include dark green vegetables, beans, legumes, citrus fruits and organ meats\textsuperscript{1,14}. While folate can be synthesized by colonic bacteria\textsuperscript{15} and absorbed across the large intestine\textsuperscript{16,17}, mammals cannot synthesize enough folate to maintain biological functions and must therefore obtain additional folate from their diet\textsuperscript{18}. Its bioavailability of naturally occurring folate in the diet varies by food source and preparation\textsuperscript{14}. Approximately 50-75\% of naturally occurring folate is lost due to food harvesting, storage, processing and preparation\textsuperscript{14}. Furthermore, folate activity is lost at low pH levels as it is oxidized\textsuperscript{1}. The fully oxidized and monoglutamylated structure of folic acid is both stable and bioavailable, making it suitable for supplements and fortified foods\textsuperscript{5}. Bioavailability of folic acid when taken with food and on an empty stomach is approximately 85\% and 100\%, respectively\textsuperscript{14}.

2.1.2 Absorption and Metabolism

Folates are absorbed primarily in the duodenum and jejunum of the small intestine\textsuperscript{19} as well as across the colon\textsuperscript{20,21} based on the presence of reduced folate carrier (RFC) and proton coupled folate transport (PCFT). Active transport is required for monoglutamyl folate, but in concentrations of >10 µmol/L folate is absorbed by passive diffusion in a nonsaturable manner. Additionally, folic acid supplementation can saturate and overwhelm the capacity of dihydrofolate reductase (DHFR) to reduce and methylate folic acid to 5-methyltetrahydrofolate (5-MTHF)\textsuperscript{22}. It is not uncommon to attain high levels of folic acid consumption owing to supplementation and current fortification practices. Hence, appreciable amounts of unmetabolized folic acid (UMFA) have been reported to appear in circulation\textsuperscript{21–26}.

Folate in its polyglutamylated form cannot enter the enterocyte because it contains more than three glutamate residues\textsuperscript{27,28}. Thus polyglutamylated folate requires glutamate carboxypeptidase II (GCPII), an exopeptidase anchored to the intestinal apical brush border membrane, to cleave glutamate chains until folate is in its monoglutamylated form\textsuperscript{27,28}.
Transportation of folate into the cell membrane is mediated by two main classes of systems: transmembrane carriers and folate-binding protein-mediated systems. Types of transmembrane carriers are the RFC, PCFT, a family of low-affinity membrane carrier multidrug resistance-associated proteins (MRPs), and mitochondrial and lysosomal folate transport. Types of folate-binding protein-mediated systems include three high-affinity folate receptors (FR) (α, β, and γ) (Figure 2.1.2).

PCFT and RFC are highly expressed in the small intestine, and are the main transport carriers for monoglutamyl folate. PCFT exhibits equal affinity for both reduced and oxidized forms of folate, and a higher affinity for 5-MTHF at a low pH of <6.5 that is consistent with the microenvironment of the small intestine. RFC transports reduced folate at optimal physiological pH (pH > 6.5). It is ineffective for transporting folic acid. MRPs have low affinity for folate but have a high capacity for exporting folate out of tissues. FRs exhibit affinity for all forms of folate; however, their affinity is highest for folic acid. Both FR α and β are expressed in the gut mucosa of fetal tissue and a number of adult tissues in low levels. FRs are found on the apical membrane of the enterocyte and thus function to transport folate into the cell. Unlike transmembrane carriers like PCFT and RFC in the apical and basolateral membranes, FRs do not transport folate in both directions.

5-MTHF is the main form of folate found in portal circulation and is taken up by the liver. 5-MTHF is metabolized to polyglutamylated derivatives in the liver where it is either retained for storage or converted back to the monoglutamate form before being released into the blood or bile. Once monoglutamylated folate enters the cell via the aforementioned transport systems, folate is converted back to its polyglutamylated form via folypolyglutamate synthase (FPGS) in order to retain folate in the cell. Polyglutamylated folates are better substrates for one-carbon metabolic reactions. Before leaving the cell
via RFC and PCFT, terminal glutamate residues of folypolyglutamates are cleaved to create monoglutamyl folate by \(\gamma\)-glutamyl hydrolase (GGH), which contributes to the maintenance of intracellular folate homeostasis.

**Figure 2.1.2. Absorption and intracellular uptake of folates.** Before entering the enterocyte of the small and large intestines, polyglutamated folates require cleavage by GCPII to one glutamate residue while monoglutamyl folate is actively transported by PCFT, RFC and FR. Once inside the cell, FPGS adds glutamate residues to retain polyglutamylated folate for intracellular one-carbon transfer reactions. For folate export out of the cell, GGH cleaves glutamate residues from polyglutamylated folate to a monoglutamylated form, which can then be transported out of the cell by RFC, PCFT and MRPs. Folate transporters have varying functions depending on the location and intraluminal and intracellular pH. [GCPII, glutamate carboxypeptidase II; PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; FR, folate receptor; FPGS, folypolyglutamyl synthase; MRP, multidrug resistance-associated proteins.] Adapted from Kim 2007\(^{28}\) and reprinted with permission from Plumptre 2016\(^{33}\).
2.1.3 Biochemical Functions

Folate functions primarily to transfer one-carbon units involved in nucleotide biosynthesis, the remethylation of homocysteine (Hcy) as part of the methionine cycle and biological methylation reactions.\textsuperscript{28, 29} In \textit{de novo} nucleotide biosynthesis, folate is a cofactor that is essential for DNA synthesis, stability, integrity and repair.\textsuperscript{29, 14} In addition to methylation of DNA (for gene expression regulation and gene stability), folate is also involved in methylation of proteins (for post-translational modifications) and lipids (for synthesis).\textsuperscript{35}

Folate-mediated one-carbon metabolism is generally believed to be compartmentalized occurring at specific areas intracellularly (cytoplasm, mitochondria, nucleus).\textsuperscript{36} Cytosolic folates such as tetrahydrofolate (THF) and 5-MTHF are mainly associated with purine and thymidylate synthesis and biological methylation reactions. Mitochondrial folates such as THF and 10-formylTHF are primarily related with the serine/glycine cycle.\textsuperscript{7} Nuclear folates are shown to mediate thymidylate synthesis, but are also involved in the serine/glycine cycle.\textsuperscript{36}

For naturally derived folates that have entered the cell, these folates can directly participate in one-carbon metabolism.\textsuperscript{34, 17, 18} This is because it exists mainly in the forms of 10-formylTHF and more predominantly 5-MTHF.\textsuperscript{34} However, for folic acid, it must be reduced to dihydrofolate (DHF) then to THF by DHFR, and methylated to 5-MTHF. This occurs primarily in the liver and to a lesser extent in the small intestine.\textsuperscript{22} Due to the limited enzymatic capacity of DHFR to reduce folate, high intakes of folic acid result in the appearance of unaltered or UMFA in circulation.\textsuperscript{19} This in turn has been shown to compete with active forms of folate for folate transporters, binding sites, and folate-related enzymes.\textsuperscript{34, 17, 28}
5-MTHF is critical in both DNA synthesis and DNA methylation (Figure 2.1.3). 5-MTHF is synthesized from 5,10-methyleneTHF in a nonreversible reaction by methylenetetrahydrofolate reductase (MTHFR)⁴⁰. 5,10-methyleneTHF donates methyl groups for de novo pyrimidine synthesis through a catalyzed reaction by thymidylate synthase (TS) from deoxyuridine-5-monophosphate (dUMP) to deoxythymidine-5-monophosphate (dUTP or thymidylate). As a result of the nonreversible methylation reaction by TS, 5,10-methyleneTHF is oxidized to DHF and thymidylate can be used for DNA synthesis. Subsequently, THF is produced from DHF by DHFR and can be converted to 5,10-methyleneTHF by vitamin B₆-dependent serine hydroxymethyltransferase (SHMT), and then to 5-MTHF by MTHFR. 5,10-methyleneTHF can also be oxidized to 10-formylTHF for de novo purine synthesis (Figure 2.1.3).

For DNA methylation reactions, 5-MTHF transfers a single methyl group to Hcy catalyzed by vitamin B₁₂-dependent methionine synthase (MS) to generate methionine⁴¹,⁴². Methionine is converted to SAM through the action of adenosine triphosphate (ATP) and methionine adenosyltransferases (MAT1A and MAT2A). DNA methyltransferases 1, 3a and 3b (DNMTs) then transfers a methyl group to DNA from SAM⁴³,⁴⁴. SAM is a universal methyl donor for over 100 biological methylation reactions⁴⁵. During this process, S-adenosylhomocysteine (SAH) is produced and converted to Hcy by SAH hydrolase and allows the methylation cycle to start again⁴⁶. Another mechanism to remethylate Hcy is through a folate-independent pathway involving betaine or choline. After the methyl group is donated from 5-MTHF, again THF is converted to 5,10-methylene THF by SHMT. 5,10-methylene THF is necessary throughout the one-carbon metabolism pathway and can be used to regenerate methionine or directed towards nucleotide biosynthesis (Figure 2.1.3).
Figure 2.1.3. Simplified diagram of the folate metabolism pathway. GCPII, glutamate carboxypeptidase II; FR, folate receptor; PCFT, proton-couples folate transporter; RFC, reduced folate carrier; FPGS, folylpolyglutamate synthase; GGH, γ-glutamyl hydrolase; DHF, dihydrofolate; THF, tetrahydrofolate; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; BHMT betaine-homocysteine S-methyltransferase; MS, methionine synthase, MSR/MTRR, methionine synthase reductase; MAT 1a, 1b, methionine adenosyltransferase; SAHH, SAH hydrolase; MTHFR, methylenetetrahydrofolate reductase; DNMT1, 3a, 3b, DNA methyltransferases; MBD2, methyl-CpG binding domain protein 2; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; CBS, cystathionine-β-synthase; CTH, γ-cystathionase; CpG, cytosine-guanine dinucleotide sequence; dUMP, deoxyuridine-5-monophosphate; dTMP, deoxythymidine-5-monophosphate; FAD, flavin adenine dinucleotide; FADH2, 1,5-dihydro-flavin adenine dinucleotide. Each filled triangle represents a glutamate, which is linked via a peptide bond to form polyglutamated folate. Each filled circle represents reduced or oxidized folates or folic acid. Adapted and modified with permission from John Wiley and Sons."
2.1.4 Folate Intake Requirements

Dietary folate intake is expressed as dietary folate equivalents (DFEs) in order to account for the lower bioavailability of natural folate compared to folic acid. Specifically, $1 \mu g$ DFE = $1 \mu g$ of food folate = $0.6 \mu g$ of folic acid from fortified foods or supplements taken with meals = $0.5 \mu g$ of a supplement taken on an empty stomach$^{17}$. Hence, DFEs can be calculated by the following equation$^{14}$:

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

Dietary Reference Intakes (DRIs) established by the Institute of Medicine$^{14}$ are scientifically evidence-based reference values used for dietary intake assessment and health planning for populations and individuals. The DRIs vary by sex, age and have separate requirements for particular populations such as pregnant and lactating subpopulations. DRIs consist of four measures: Estimated Average Requirement (EAR), the Recommended Dietary Allowance (RDA), the Tolerable Upper Limit (UL), and the Adequate Intake (AI).

Briefly, the EAR is the daily nutrient intake value that is estimated to meet the requirements of 50% of the population. The RDA is the average daily intake required to meet the requirement of approximately 97-98% of the population, and it can be calculated by using the EAR + 2 standard deviations. If the EAR is not available due to insufficient data, the RDA can be calculated using the EAR x 1.2 as this assumes 10% coefficient of variation for the EAR, and is therefore equal to 120% of the EAR. When not enough information is available to calculate an EAR and RDA, the AI is used as an average daily recommendation based on observed or experimental estimates of nutrient intakes from a healthy population$^{48}$. The UL is the highest level of daily nutrient intake for individuals without risk of adverse health effects. For most nutrients, as the intake increases above the UL, the risk of adverse health effects increases$^{14}$. 
DRIs for folate are based on observational and experimental studies. The EAR and RDA for folate for both males and females 19 years old and above are 320 and 400 µg/d DFEs, respectively. For pregnant and lactating women, the EAR and RDA are increased to 520 µg/d and 600 µg/d DFE, respectively (Table 2.1.4). An additional 400 µg/d of folic acid either in the form of supplements or fortified foods is recommended 2-3 months prior to conception and during pregnancy to reduce the risk of neural tube defects. For pregnant women, the increase in requirement is due to multiple fetal and maternal physiological changes that increase metabolic demands for folate. Ensuring adequate folate intake is critical for maintaining one-carbon transfer reactions including DNA synthesis and repair and biological methylation reactions necessary for optimal maternal health and fetal growth and development. For lactating women, the increase in requirement is necessary to replace the amount of folate secreted daily in the breast milk, thereby ensuring optimal folate status.

There is no UL for naturally occurring folate, but the UL is set at 1000 µg/d for folic acid for adults 19 years and older and pregnant and lactating women (Table 2.1.4). Although studies are limited, the UL for folic acid was set at this level based on suggestive evidence that excessive amounts of folic acid may mask B₁₂ deficiency, thereby allowing irreversible neurological damage to continue to progress. This phenomenon where high folate masks B₁₂ deficiency is called the methyl folate trap or ‘methyl trap’. In this scenario, folate is trapped in the form of 5-MTHF due to the constant and irreversible conversion of 5,10-methyleneTHF to 5-MTHF by MTHFR. With low B₁₂, MS activity is reduced and subsequently leads to a decrease in remethylation of methionine from Hcy. The remethylation of methionine works by the transfer of a methyl group from 5-MTHF to homocysteine to form THF (Figure 2.1.3). During this process, the regeneration of methionine from homocysteine by MS is also diminished, resulting in reduced methylation activity. In addition to masking B₁₂ deficiency, excess folate has been implicated in resistance to antifolate drugs used in cancer treatment and inflammatory disorders and reduced natural killer cell cytotoxicity.
Table 2.1.4. 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.

2.1.5 Biomarkers of Folate

2.1.5.1 Folate Status

Serum or plasma is a short-term indicator of folate status as it represents recent dietary intake, while red blood cell (RBC) folate concentrations reflect tissue stores. Together, they are used to measure systemic folate status. Plasma folates are monoglutamylated folates with variations in oxidation state and one-carbon substitutions. 5-MTHF predominates in the serum/plasma, generally accounting for >80% of total plasma folates. Plasma also contains UMFA and non-5-methylated reduced folates usually in small concentrations. RBC folates have higher folate concentrations than in the serum or plasma and are mostly 5-MTHF polyglutamates, except in persons with MTHFR677T polymorphism where a proportion of 5-MTHF is converted to formyl-folates. Measurement is also more complex than with serum folate as polyglutamates need conversion to monoglutamates before analysis. RBC folate measurement is not influenced by recent folate intake as RBCs have a lifespan of approximately 120 days, and is therefore indicative of long term (~3 months) folate intake and tissue stores. It is for this reason that RBC folate measurement is considered the gold standard for determining folate status.

Plasma homocysteine is a functional indicator of folate status; however, it is not a specific marker as it is also influenced by the status of vitamins B₂, B₆ and B₁₂. 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 reflects high intake levels of folic acid. UMFA is found to be increasingly prevalent in blood samples of many populations, and is not limited to countries with mandatory folic acid fortification\(^5^9\). Observational studies have found detectable levels of UMFA in children, adolescents, and adults\(^5^7,^6^0\), older adults\(^6^1\), pregnant women and umbilical cord blood\(^2^3\) and in 4-day old infants\(^6^2\).

### 2.1.5.2 Analysis of Folate in Blood and Other Biological Fluids

There are three main methods for analysing folates in serum, plasma and RBCs, and other biological fluids. They are accomplished by the microbiological, protein-binding and chromatographic assays as shown in

<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. rhamnosus) is proportional to</td>
<td>-Gold standard of overall folate status</td>
<td>-Does not distinguish between individual folate</td>
</tr>
<tr>
<td></td>
<td>amount of folate in sample</td>
<td>-Very sensitive</td>
<td>forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Inexpensive</td>
<td>-Low precision</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Measures all biologically active forms equally</td>
<td>-Can be affected by antibiotics</td>
</tr>
<tr>
<td>Protein-binding</td>
<td>Folate binding proteins &quot;extract&quot; folate from samples,</td>
<td>-Quick results</td>
<td>-Narrow detection range</td>
</tr>
<tr>
<td></td>
<td>can be radio- or nonradio-labelled</td>
<td>-High-throughput analysis</td>
<td>-Binding proteins respond differently depending on</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Good precision</td>
<td>forms of folate in sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Low cost</td>
<td></td>
</tr>
<tr>
<td>Chromatographic</td>
<td>Individual forms of folate are separated and quantified</td>
<td>-Can measure individual folate forms</td>
<td>-Extensive sample cleaning</td>
</tr>
<tr>
<td></td>
<td>based on their interaction with the adsorbent material.</td>
<td>-Highly selective and sensitive</td>
<td>-Expensive specialized equipment</td>
</tr>
<tr>
<td></td>
<td>May be quantified by measuring the mass to charge ratio.</td>
<td></td>
<td>-Trained personnel</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Interconversions of folate forms needed for correct</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>interpretation of data</td>
</tr>
</tbody>
</table>

Table modified from Plumptre, 2016 thesis\(^3^3\).
2.1.5.3 Common Cutoffs Used to Define Deficient and High Folate Status

The only formally accepted RBC cutoff is for folate deficiency, and it is defined as having a RBC folate concentration less than 305 nmol/L (140 ng/mL), which is the level at which hematological (e.g. macrocytic anemia) and metabolic (e.g. rise in homocysteine concentrations) abnormalities begin to appear\cite{14,64}. For serum folate, a concentration less than 7 nmol/L (3 ng/mL) is also an indicator of folate deficiency; however, this is less commonly used as serum folate is sensitive to recent intakes\cite{14}.

Due to increasing folate status arising from fortification and supplement use, several cutoffs for high folate status have been suggested; however, no formally accepted cutoffs exist to date. A RBC folate concentration of 1360 nmol/L using Bio-Rad immunoassay is considered high based on the 97th percentile of RBC folate concentrations using the 1999-2004 National Health and Nutrition Examination Survey (NHANES) data\cite{10}. Using the 2007-2009 Canadian Health Measures Survey (CHMS), three upper RBC folate concentrations cutoffs have been to facilitate future research examining the impact of high folate status on health outcomes – 1450, 1800, and 2150 nmol/L\cite{65}. These were estimated from post-fortification 2005-2010 NHANES data in increments of 305 nmol/L (adjusted from the Immulite 2000 immunoassay to microbiologic assay). Based on the CHMS data, 16%, 6% and 2% of sampled Canadian population were at or above the 1450, 1800, and 2150 nmol/L, respectively\cite{65}.

1. Summary of Cutoffs

Low cutoffs have been more consistent as they are mainly based on deficiency levels (Table 2.1.5.3). A few proposed high cutoffs have been proposed based on epidemiological studies. The ‘low’ cutoffs are 305, 906 and 1000 nmol/L. The 305 nmol/L is the most widely accepted cutoff for folate deficiency, below which hematological (e.g. macrocytic anemia) and metabolic (e.g. an increase in plasma homocysteine concentrations) indicators begin to appear\cite{14,64}. For serum folate, a concentration less than 7 nmol/L (3
ng/ml) is also an indicator of folate deficiency; however, this is a less reliable marker as serum folate is sensitive to recent folate intakes\textsuperscript{14}. A cutoff of at least 906 nmol/L RBC folate concentration is conventionally used as the level of maximal protection to prevent NTDs. A large Irish case-control study (n=56,000 pregnant women) found that the risk for NTD decreased by eight-fold in those with RBC folate concentrations >906 nmol/L, compared those with RBC folate concentrations <305 nmol/L\textsuperscript{66}. This study also reported a dose-response relationship between RBC folate concentrations and the risk of NTD-affected pregnancy\textsuperscript{66}, where the risk of NTD begins to decrease at 906 nmol/L and continues to do so until it reaches a plateau at 1292 nmol/L\textsuperscript{66}. Although the 906 nmol/L is the cutoff conventionally used, two large studies in China (total n=249,045) demonstrated that a cutoff above 1000 nmol/L substantially attenuated risk for NTD\textsuperscript{67}. Applying this cutoff via logistic model regression to various large-scale population data like the NHANES (2005 - 2010), the 1000 nmol/L cutoff further reduced the risk for NTD to a level below moderate risk (<8 NTDs per 10,000 births)\textsuperscript{67}. Hence, a cutoff above 1000 nmol/L is proposed cutoff is suggested to be an optimal RBC folate concentration for NTD prevention. The association of 'high' RBC folate cutoffs with adverse health outcomes has not been established. Nevertheless, these cutoffs have been proposed to define high folate status\textsuperscript{68} (Table 2.1.5.3). The 1090 nmol/L cutoff is based on RBC folate concentrations from individuals consuming 2.1 mg DFEs or of the combined intake of 400 µg DFEs (RDA excluding for pregnant and lactating women) and 1000 µg folic acid supplement\textsuperscript{69}. The 1360 nmol/L cutoff represents the 97\textsuperscript{th} percentile according to the 1999-2004 National Health and Nutrition Examination Survey (NHANES) data, using Bio-Rad immunoassay\textsuperscript{10}. Based on a more recent 2005 - 2010 NHANES, the 1820, 2150, 2490 nmol/L cutoffs represent the 90\textsuperscript{th}, 95\textsuperscript{th} and 97.5\textsuperscript{th} percentile of the 2005-2010 NHANES cutoffs, adjusted to microbiologic assay\textsuperscript{70}. In the 2007-2009 CHMS, three upper RBC folate concentrations in increments of 305 nmol/L were selected and estimated from the 2005-2010 NHANES cutoffs ~ 1450, 1800, and 2150 nmol/L\textsuperscript{65}. 
Table 2.1.5.3. A summary of folate cutoffs

<table>
<thead>
<tr>
<th>RBC folate cutoff (nmol/L)</th>
<th>Rationale</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>305</td>
<td>Considered for folate deficiency; hematological (e.g. macrocytic anemia) and metabolic indicators (e.g. increasing plasma homocysteine concentrations).</td>
<td>IOM 1998</td>
</tr>
<tr>
<td>906</td>
<td>Conventionally used as the level of maximal protection to prevent NTDs based on a large Irish case-control study.</td>
<td>Daly et al. 1995</td>
</tr>
<tr>
<td>1000</td>
<td>A more recent measure based on two large and recent studies in China.</td>
<td>Crider et al. 2014</td>
</tr>
<tr>
<td>High:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1090</td>
<td>Based on the relationship between RBC folate and intakes in DFEs.</td>
<td>MacFarlane et al. 2011</td>
</tr>
<tr>
<td>1360</td>
<td>97&lt;sup&gt;th&lt;/sup&gt; percentile of the 1999 - 2004 NHANES</td>
<td>Colapinto et al. 2011</td>
</tr>
<tr>
<td>1820, 2140, 2490</td>
<td>90&lt;sup&gt;th&lt;/sup&gt;, 95&lt;sup&gt;th&lt;/sup&gt; and 97.5&lt;sup&gt;th&lt;/sup&gt; percentile of the 2005 - 2010 NHANES</td>
<td>Pfeiffer et al. 2012</td>
</tr>
<tr>
<td>1450, 1800, 2150</td>
<td>Estimated from 2005 - 2010 NHANES in increments of 305 nmol/L.</td>
<td>Colapinto et al. 2015</td>
</tr>
</tbody>
</table>
2.1.6 Folate and Health

2.1.6.1 Folate in Health and Disease

Folate has been shown to affect human health and disease through its role in purine and thymidylate synthesis and biological methylation reactions. These one-carbon transfer reactions play a critical role in nucleotide biosynthesis and repair, genomic stability, and regulation of gene expression. Adverse health outcomes associated with both inadequate and excess intakes of folate have been summarized in Table 2.1.6.1.

Table 2.1.6.1. Health outcomes associated with inadequate and excess folate

<table>
<thead>
<tr>
<th></th>
<th>I. Inadequate folate</th>
<th>II. Excess folate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convincing</td>
<td>• Megaloblastic anemia</td>
<td>• Masking B₁₂ deficiency</td>
</tr>
<tr>
<td>Probable</td>
<td>• Coronary heart disease</td>
<td>• Cancer progression</td>
</tr>
<tr>
<td></td>
<td>• Stroke</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Cancer initiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Cognitive impairment</td>
<td></td>
</tr>
<tr>
<td>Possible/Insufficient</td>
<td>• Neuropsychiatric disorders</td>
<td>• Antifolate drug resistance</td>
</tr>
<tr>
<td></td>
<td>• Other congenital disorders</td>
<td>• Decreased natural killer cell cytotoxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Increased risk of obesity, insulin resistance in offspring</td>
</tr>
</tbody>
</table>

Table modified from Plumpre 2016 thesis.

1. Folate Deficiency

Evidence to support the most purported adverse health outcomes associated with folate deficiency except for megaloblastic anemia and NTDs is not consistent and is tenuous in the literature.

Megaloblastic Anemia

Megaloblastic anemia is a hematological indicator of folate deficiency. It has been characterized by the appearance of hypersegmented neutrophils and has been associated with low RBC folate concentrations.
Clinically, common manifestations of megaloblastic anemia start with pallor of the skin and can progress to fatigue, weakness, shortness of breath and palpitations\textsuperscript{71}. The evidence supporting the link between folate deficiency and megaloblastic anemia has been consistent\textsuperscript{14}, and has thus been used as a basis for establishing national folate deficiency cutoffs in Canada and the US\textsuperscript{64}.

Probable

*Coronary Heart Disease and Stroke*

Epidemiological and clinical studies have investigated the relationship between high homocysteine levels and the risk of coronary heart disease and stroke; however, the evidence is inconsistent and equivocal\textsuperscript{72}. The Homocysteine Studies Collaboration that pooled 30 prospective and retrospective studies reported that for every 25\% reduction in homocysteine concentration (approximately 3\(\mu\)mol/L), an 11\% decreased risk of coronary heart disease and a 19\% decreased risk of stroke were observed\textsuperscript{72,73}. In contrast to the results from observational epidemiologic studies, most placebo-controlled randomized clinical trials and their systematic reviews and meta-analyses have reported a null effect of folic acid supplementation with or without other B vitamins on coronary heart disease risk \textsuperscript{4,74–78}. However, these studies have suggested that folic acid supplementation may exert a protective effect on stroke risk \textsuperscript{78–81}.

*Cancer Initiation and Progression*

The role of folate in cancer development and progression has been highly controversial. A large body of epidemiologic studies collectively suggest an inverse association between suboptimal folate status and the risk of several human malignancies including cancer of the lungs, oropharynx, esophagus, stomach, colorectum, pancreas, cervix, endometrium, ovary, bladder, prostate, skin, and breast as well as neuroblastoma and leukaemia \textsuperscript{82–84}. However, the precise nature and magnitude of this purported relationship have not been unequivocally established and may be influence by the baseline folate status of the population.
studied. The role of folate in carcinogenesis is best studied for colorectal cancer (CRC). Collectively observational epidemiologic studies and their systematic reviews and meta-analyses have indicated a 20 – 40% reduction in the risk of CRC in subjects with the most adequate folate status (assessed by dietary folate intake or blood measurement of folate biomarkers) compared to those with the lowest. However, several placebo-controlled randomized clinical trials have reported an increase, decrease, or null effect of folic acid supplementation on the risk of CRC or recurrent adenomas (a well-established precursor).

Animal studies have suggested dual modulatory effects of folate on the initiation and progression of CRC depending on the dose and stage of cell transformation at the time of folate intervention. Folate deficiency has been shown to increase, while modest levels folic acid supplementation reduces the risk of neoplastic transformation in normal tissues. However, it appears that excessive amounts of folic acid supplementation can paradoxically increase the risk of neoplastic transformation in normal tissues. Conversely, in preneoplastic and neoplastic lesions, folate deficiency has been shown to suppress, whereas folic acid supplementation promotes the progression of these lesions. Although the mechanisms to explain folate’s dual effects on CRC initiation and progression have yet to be clearly elucidated, several plausible mechanisms relating to nucleotide biosynthesis and DNA methylation have been proposed and extensively studied.

Neuropsychiatric Disorders & Cognitive Impairment

Although not uniformly consistent, epidemiologic studies have suggested an inverse association between folate status and the risk of cognitive impairment, dementia and depression. Furthermore, randomized clinical trials have suggested a possible protective effect of folic acid supplementation with and without other B vitamins on cognitive decline.
Other Congenital Disorders

In contrast to the well-established inverse relationship between maternal folate status and the risk of NTDs, the relationship between maternal folate status and the risk of other congenital defects has been equivocal. Several randomized clinical trials and observational studies have found a decrease in risk of congenital defects with multivitamin supplementation containing folic acid.

II. Excess Folate

Due to the increased folate status resulting from folic acid supplementation, in addition to voluntary and mandatory folic acid fortification in many countries, there has been increasing interest in potential adverse health outcomes associated with excess folate and folic acid.

B<sub>12</sub> Masking

B<sub>12</sub> masking is a scenario of excess folate and deficient B<sub>12</sub> status. With low B<sub>12</sub>, MS activity is reduced resulting in reduced remethylation of homocysteine to produce methionine. Folic acid supplementation negates the adverse hematological effects (i.e. megaloblastic anemia) of low B<sub>12</sub>, thus masking and delaying diagnosis of B<sub>12</sub> deficiency. As a result, there remains a risk for the progression of B<sub>12</sub> deficiency-associated neurological conditions (e.g., subacute combined degeneration). Furthermore, B<sub>12</sub> has been important for maintaining cognitive function in the aging population, and B<sub>12</sub> masking has been shown to increase the risk of cognitive impairment in the elderly.

Cancer Progression

The effect of excess folate on cancer progression has been generally inconsistent and conflicting. Animal studies suggest a dual modulatory role of folate in cancer progression. Although folic acid supplementation may protect against neoplastic transformation in normal tissues, it appears to promote the progression of
established (pre)neoplastic lesions. Several biologically plausible mechanisms relating to the folate’s role in one-carbon transfer reactions exist to explain the tumor promoting effect of folic acid supplementation. Most likely, folic acid supplementation provides nucleotide precursors to rapidly replicating cells such as neoplastic cells for accelerated progression. Gene silencing of tumor suppressor or mismatch repair genes via CpG island promoter DNA methylation has been proposed as another plausible mechanism for the tumor promoting effect associated folic acid supplementation. In large-scale placebo-controlled randomized clinical trials that investigate folic acid supplementation and risk of recurrent adenomas as the primary endpoint; an increase, decrease, or a null effect was observed (121,129,130). The Aspirin/Folate Polyp Prevention Study associated folic acid supplementation with a 67% increased risk of recurrent advanced adenomas with a high malignant potential in individuals with prior colorectal adenomas. In the 12-year French SUViMAX prospective study (n=12741), dietary folate intake and RBC folate concentration was associated with an increased risk of overall skin cancer. Clinical trials with cancer incidence or mortality as a secondary endpoint have shown either a tumour promoting or null effect on cancer outcomes with folic acid supplementation. A Norwegian study that investigated the effect of folic acid (800 µg/d) and B vitamins (B₁₂ 400 µg/d) supplementation on ischemic heart disease as the primary outcome found an overall increase in cancer incidence by 21% and mortality by 38% over a 36-month period. In contrast, a meta-analysis of eight randomized control trials of folic acid supplementation on cardiovascular disease outcomes as the primary endpoint found no significant effects on overall cancer incidence and mortality over a 5-year period. Similarly, a meta-analysis of 13 trials (studies investigating folic acid supplementation with cardiovascular risk as the primary endpoint and CRC risk in patients with previous adenomas) found no overall effect of folic acid supplementation on CRC risk. Nevertheless, a non-significant trend toward an increased risk of CRC was observed in the 3 trials involving patients with previous adenomas and in all 13 trials. Two ecologic studies on the trend of CRC incidence post-folic acid fortification era found increased CRC rates in the US, Canada and Chile, suggesting that folic
acid fortification may have been responsible for this observation\textsuperscript{104,105}. On the other hand, two large US prospective studies conducted in the postfortification era found that a moderate consumption of folate reduced the risk of CRC, and no tumor promoting effect was associated with folic acid supplementation or fortification\textsuperscript{106,107}.

\textit{Resistant to Antifolate Drugs}

Antifolate drugs are used against arthritis\textsuperscript{108,109}, inflammatory conditions, and cancer\textsuperscript{110–112} as they disrupt intracellular folate metabolism and related one-carbon metabolism. In an environment with excess folate, the potency or efficacy of antifolate drugs may be diminished and a resistance to these drugs may emerge\textsuperscript{45,110,111}.

\textit{Immune System}

Excess folate (mostly supplemental folic acid) has also been linked with diminished natural killer (NK) cell cytotoxicity. NK cells are immune cells with important defence mechanisms against viral infections and cancer cells\textsuperscript{111}. A study in postmenopausal women found that a high folate diet and folic acid supplementation were associated with reduced NK cell cytotoxicity in comparison to the control\textsuperscript{114}. Women with low combined dietary folate and folic acid supplementation were also reported to have 23\% lower NK cell cytotoxicity\textsuperscript{114}. This inverse U-shaped curve was more pronounced among women over 60 years old. Furthermore, in the 78\% of women with detected UMFA, their NK cell cytotoxicity was 6.2\% lower compared with women without plasma FA present\textsuperscript{114}. Similarly, a recent non-controlled intervention (n=30) in Brazil observed a reduction in cytotoxic capacity and number of NK cells with 5000 µg folic acid supplementation and increased UMFA concentrations after 45 and 90 days\textsuperscript{55}. In an animal model, female mice were fed a 20 x RDA folic acid diet for 3 months were found to have lower NK cytotoxicity compared to control mice (1 x RDA folic acid)\textsuperscript{54}.
Several studies have investigated prenatal folic acid supplementation with the risk of adiposity and insulin resistance in the offspring. Animal studies have shown more consistent effects on these outcomes in offspring from mothers supplemented with folic acid alone, whereas findings are conflicting in studies where folic acid supplementation was used in combination with other B vitamins, including B₁₂ (See Section 2.2.6.2). Two rodent studies have shown that pups born to folic acid supplemented dams have a higher birth weight\textsuperscript{115,116} and are more insulin resistant\textsuperscript{116}. In a sheep model, periconceptional folate, B₁₂ and methionine that was restricted in dams and ova from these mothers, were fertilized \textit{in vitro} and transferred to surrogate dams with normal methionine status\textsuperscript{117}. The offspring, especially males, were found to be obese and insulin resistant compared to males from ewes in the non-restricted diet\textsuperscript{117}. In humans, the Pune Maternal Nutrition Study from India was the first to find an increased risk of insulin resistance and obesity in children at 6 years of age associated with high maternal folate and low B₁₂ status\textsuperscript{13} at mid-pregnancy (18 weeks gestation). A study in Myosore, Southern India reported similar findings to the Pune study in that higher maternal folate status was associated with higher offspring insulin resistance children at 9.5 and 13.5 years old\textsuperscript{118}. However, unlike the Pune study, the Myosore study did not find an interaction between maternal folate and B₁₂ status. Another interesting finding of the Myosore study was that children at 5 years old had fasting glucose concentrations associated positively with folate and negatively with B₁₂ blood concentrations\textsuperscript{118}. Since the Myosore study sampled mothers in late gestation (30 weeks), the window for detecting the effects of B₁₂ on insulin resistance may have passed, and this may be a reason for the observed lack of interaction between folate and B₁₂\textsuperscript{118}. Further analysis from the Pune study has revealed that late gestation (28 weeks) folate concentrations and mid-gestation (18 weeks) B₁₂ concentrations are the strongest predictor of insulin resistance in the offspring\textsuperscript{13}.
In summary, both folate deficiency and excess have been shown to affect human health. Due to the significantly increased folate status resulting from folic acid fortification and supplemental practices and virtually non-existent folate deficiency in the North American population, there has been a shift in research focus on investigating the effects of a high folate status on health and disease outcomes.

2.1.6.2 Folic Acid Fortification

As mentioned previously in Section 2.1.6.1, there are several adverse health outcomes related to folate deficiency including the increased risk of NTDs. A large body of evidence has consistently suggested a decrease in NTD risk with periconceptional use of folic acid\textsuperscript{91,119–121}. It is primarily for this reason that the Canadian and US governments mandated folic acid fortification in 1998 and recommended women at reproductive age to supplement with folic acid\textsuperscript{122,123}. The addition of 150 µg folic acid/100 g white wheat flour and 200 µg folic acid/100 g cornmeal and enriched pasta (140 µg folic acid/100 g for all three grains in the US) led to an approximately 50% decrease in NTD prevalence in Canada and the US, deeming folic acid fortification a public health policy success\textsuperscript{124–127}.

Since then, several studies have suggested an over-fortification of the food supply beyond the recommended 100 – 200 µg\textsuperscript{128}, and that even these high levels are thought to be an underestimate of the actual amount of folic acid in the fortified products\textsuperscript{129}. Epidemiological studies have suggested that mandatory folic acid fortification has significantly contributed to the increase in serum and RBC folate concentrations evident in the North American population post folic acid fortification\textsuperscript{126,128}. Comparison of blood folate measures from NHANES pre (1988-1994) and post (1999-2004) folic acid fortification indicate a decline in the prevalence of folate deficiency by 21% as evaluated by serum folate (\(<1%\) for serum folate \(<6.8\) nmol/L) and 38% as evaluated RBC folate (5% for RBC folate \(<305\) nmol/L) concentrations\textsuperscript{10}. An increase in serum and RBC folate concentrations by 119-161% and 44-64%, respectively, was also observed. This translates to an
increase of RBC concentrations from 398 to 636 nmol/L \(^{10}\). More recently, the Centre for Disease Control (CDC) and Prevention’s Second Nutrition Report for 2003-2006 indicated that less than 1% of the US population was folate deficient\(^{130}\). Canadian data illustrate similar trends with 96.3% of the population from 2007-2009 with adequate status evident by RBC folate concentrations. Interestingly, 40% of the Canadian population had RBC folate concentrations above the high cutoff (1360 nmol/L) set as the 97.5\(^{th}\) percentile of the NHANES 1999-2004 data\(^{131}\). Applying the most recent proposed high cutoffs of 1450, 1800 and 2150 nmol/L recommended to evaluate the impact of high RBC folate on health outcomes, 16%, 6% and 2%, respectively, of participants in the 2007-2009 CHMS, had high RBC values\(^{65}\). The prevalence of high RBC folate concentrations was higher in females, those between 60-79 years of age, overweight or obese individuals, and folic acid supplement users\(^{65}\).

Despite the reported increase in folate intakes and blood concentrations post folic acid fortification, certain groups in the population including women of childbearing age (WCBA) may be susceptible to suboptimal RBC folate concentrations. In the US, > 90% of WCBA (20-59 years old) still had RBC folate concentrations below <906 nmol/L, a purported level below which the NTD risk begins to increase, postfortification. Nevertheless, RBC mean concentrations increased from 505 to 587 nmol/L pre to post fortification\(^{132}\) in WCBA. In Canada, 22% of WCBA (15-45 years old) also had RBC concentrations below the <906 nmol/L cutoff postfortification in the most recent CHMS\(^{131}\). However, it is unknown whether these WCBA were planning a pregnancy and no data on dietary patterns and supplementation were collected. The controversy in folic acid fortification and supplementation thus persists; while high levels of folate intake and blood concentrations are evident postfortification, there is a need to continue to supplement with folic acid before and during pregnancy as a protective measure against NTDs.
2.1.6.3 Folate in Pregnancy

The effects of folate deficiency on pregnancy is discussed in Section 2.1.6.1 and summarised in Table 2.1.6.1. Briefly, folate deficiency has been associated with an increased risk of NTDs and congenital disorders.

I. Folic Acid Supplementation during Pregnancy

Studies conducted in Canada and the US report that the majority of women take supplements containing folic acid prior to and during pregnancy\textsuperscript{49,134–137}. Two recent studies in the US show that around 51% - 78% of women report taking folic acid before and during pregnancy\textsuperscript{49,134}. In Canada, a recent study reported a 60% folic acid supplement use before pregnancy, and a 93% and 89% reported use in early and late pregnancy\textsuperscript{135}. Compared to the amount of folic acid obtained from fortification (100 µg/day), most prenatal vitamins available on the market and consumed contain 1000 µg/d folic acid. This difference alone suggests that folic acid folic acid supplementation, more than fortification, may be driving the folate status in women. A study has also shown that the observed high serum folate levels from the non-pregnant participants of 2001-2004 NHANES were primarily due to supplemental sources of folic acid, and not from fortification of enriched cereal-grain products or ready-to-eat cereals\textsuperscript{138}.

II. High Maternal Folate Levels

There have been observational studies from a number of countries that investigated maternal blood folate levels during pregnancy\textsuperscript{13,23,49,51,59,137,139–142} (Table 2.1.6.3a). Among other countries with mandatory folic acid fortification, studies report high supplemental use before and during pregnancy in addition to mandatory fortification in Canada and the US\textsuperscript{135,143,144}. Elevated RBC folate concentrations during pregnancy have thus been evident in these studies and particularly in two recent observational studies in Canada and the US. The most recent Canadian study (the PREnatal FOlic acid exposuRe on DNA Methylation in the
newborn infant [PREFORM] study) reported high RBC folate concentrations in early and late pregnancy and increased RBC folate concentrations from first to third trimester (2417 and 2793 nmol/L respectively)\textsuperscript{143}. Furthermore, UMFA was detectable (≥ 0.2 nmol/L, range: undetectable to 244 nmol/L) in 97% of the women\textsuperscript{143}. Similarly, in the US, the 1999-2006 NHANES reported high RBC folate concentration during pregnancy, which increased from the first to the third trimester (1255, 1527 and 1773 nmol/L respectively)\textsuperscript{49}. On the contrary, an Irish randomized control trial (RCT) published this year investigated the effect of supplemental folic acid (400 µg) with fortification (100 µg folic acid/day) on UMFA in maternal and cord blood and found no significant difference in UMFA blood concentrations between the treatment and placebo groups\textsuperscript{145}. Nevertheless, the same study reported higher maternal plasma total folate concentrations in the folic-acid supplemented group compared with the placebo group\textsuperscript{145}. Overall, while folic acid supplementation has been shown to be beneficial during pregnancy and at birth, caution on the dose of periconceptional folic acid for supplementation must be considered as this may attribute to the elevated blood levels of folate in mothers in the current folic acid fortified environment.
### Table 2.1.6.3a. Summary of studies investigating maternal folate concentrations

<table>
<thead>
<tr>
<th>Location</th>
<th>FA fortification?</th>
<th>FA supplementation?</th>
<th>Sample size</th>
<th>Time of blood draw</th>
<th>Fasted samples?</th>
<th>Serum folate (nmol/L)</th>
<th>Median (range)</th>
<th>RBC folate (nmol/L)</th>
<th>Median (range)</th>
<th>Hcy (µmol/L)</th>
<th>Median (range)</th>
<th>% with detectable UMFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Voluntary</td>
<td>Yes</td>
<td>69</td>
<td>childbirth</td>
<td>No</td>
<td>13</td>
<td>(7.34, 14-41)</td>
<td>580</td>
<td>(252-1635)</td>
<td>7.1</td>
<td>(3.3-21.0)</td>
<td>90%</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes (17%)</td>
<td>201</td>
<td>childbirth</td>
<td>No</td>
<td>26</td>
<td>(14-41)</td>
<td>1627</td>
<td>(1044-2093)</td>
<td>8.3</td>
<td>(5.6-3.6)</td>
<td>44%</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>82</td>
<td>82</td>
<td>childbirth</td>
<td>Yes</td>
<td>27</td>
<td>±20</td>
<td>813</td>
<td>±475</td>
<td>5.6</td>
<td>(5.9-5.6)</td>
<td>-</td>
</tr>
<tr>
<td>Japan</td>
<td>No</td>
<td>28 wks</td>
<td>23</td>
<td>childbearing</td>
<td>Yes</td>
<td>23</td>
<td>±18</td>
<td>961</td>
<td>(736-1269)</td>
<td>5.9</td>
<td>(8.6-7.0)</td>
<td>-</td>
</tr>
<tr>
<td>India</td>
<td>No</td>
<td>14 wks</td>
<td>21</td>
<td>childbearing</td>
<td>Yes</td>
<td>19</td>
<td>±20</td>
<td>945</td>
<td>(278-2180)</td>
<td>8.6</td>
<td>(6.7-10.8)</td>
<td>-</td>
</tr>
<tr>
<td>India</td>
<td>No</td>
<td>36 wks</td>
<td>26</td>
<td>childbearing</td>
<td>Yes</td>
<td>36</td>
<td>±69</td>
<td>1185</td>
<td>(702)</td>
<td>5 (2.3)</td>
<td>(4.3-8.2)</td>
<td>-</td>
</tr>
<tr>
<td>Ireland</td>
<td>Limited</td>
<td>28 wks</td>
<td>21 (14)</td>
<td>childbearing</td>
<td>Yes</td>
<td>138</td>
<td>±20</td>
<td>1296</td>
<td>(35, 36)</td>
<td>24.9</td>
<td>(3, 4)</td>
<td>-</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>12 wks</td>
<td>20</td>
<td>childbearing</td>
<td>No</td>
<td>1280</td>
<td>±30</td>
<td>1250</td>
<td>(364, 1250)</td>
<td>20.7</td>
<td>(16-25)</td>
<td>-</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>12 wks</td>
<td>20</td>
<td>childbearing</td>
<td>No</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1380</td>
<td>(2721, 2867)</td>
<td>27.9</td>
<td>(20, 30)</td>
<td>-</td>
</tr>
<tr>
<td>Australia</td>
<td>Mandatory</td>
<td>1st</td>
<td>20</td>
<td>childbearing</td>
<td>Yes</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1,380</td>
<td>(702)</td>
<td>1</td>
<td>(0.9, 2.6)</td>
<td>-</td>
</tr>
<tr>
<td>USA</td>
<td>Mandatory</td>
<td>1st</td>
<td>20</td>
<td>childbearing</td>
<td>Yes</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1,380</td>
<td>(2702, 2867)</td>
<td>17.0</td>
<td>(5, 27)</td>
<td>-</td>
</tr>
<tr>
<td>Canada</td>
<td>Mandatory</td>
<td>1st</td>
<td>20</td>
<td>childbearing</td>
<td>Yes</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1,380</td>
<td>(2702, 2867)</td>
<td>9.0</td>
<td>(2.9, 27)</td>
<td>-</td>
</tr>
<tr>
<td>Canada</td>
<td>Mandatory</td>
<td>1st</td>
<td>20</td>
<td>childbearing</td>
<td>Yes</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1,380</td>
<td>(2702, 2867)</td>
<td>17.0</td>
<td>(5, 27)</td>
<td>-</td>
</tr>
<tr>
<td>Ireland</td>
<td>Mandatory</td>
<td>1st</td>
<td>20</td>
<td>childbearing</td>
<td>Yes</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1,380</td>
<td>(2702, 2867)</td>
<td>9.0</td>
<td>(2.9, 27)</td>
<td>-</td>
</tr>
<tr>
<td>Ireland</td>
<td>Mandatory</td>
<td>1st</td>
<td>20</td>
<td>childbearing</td>
<td>Yes</td>
<td>1, 2, 3rd trimester</td>
<td>±20</td>
<td>1,380</td>
<td>(2702, 2867)</td>
<td>9.0</td>
<td>(2.9, 27)</td>
<td>-</td>
</tr>
</tbody>
</table>

III. High Cord Blood Folate Levels

Although fewer studies have reported on cord blood folate concentrations, all studies have shown higher folate concentrations in the cord blood than in maternal blood\textsuperscript{23,51,139,140,143,145} (Table 2.1.5.3b). Four studies have reported the presence of UMFA in 55 – 100% of the cord blood samples\textsuperscript{23,61,62,143}; however, one RCT study reported only 20%\textsuperscript{145}. Notably, in RCTs, the level of folic acid supplementation and fortification in the studied population were at 400 µg/d and 100 µg/d, respectively. While in observational studies, an average folic acid intake of 400 µg to 1000 µg/d was reported. Interestingly, among the four studies reporting the occurrence of UMFA in cord blood, two were in populations without mandatory folic acid fortification and supplementation\textsuperscript{61,62}.

**Table 2.1.6.3b. 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>Hcy (µmol/L)</th>
<th>UMFA (nmol/L)</th>
<th>% with detectable UMFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>Voluntary</td>
<td>Not addressed</td>
<td>69</td>
<td>immuno</td>
<td>HPLC/MB</td>
<td>28 (15-38)</td>
<td>1027 (305-2627)</td>
<td>6 (2.3-15.3)</td>
<td>0.42 (0.0-0.60)</td>
<td>100%</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes (17%)</td>
<td>201</td>
<td>MBA</td>
<td>HPLC</td>
<td>47 (33-62)</td>
<td>2142 (1645-2549)</td>
<td>7.9±2.9</td>
<td>0.28 (0-0.51)</td>
<td>85%</td>
</tr>
<tr>
<td>Germany</td>
<td>No</td>
<td>No</td>
<td>82</td>
<td>immuno</td>
<td>LC-MS</td>
<td>61±21</td>
<td>-</td>
<td>5.4±1.9</td>
<td>10.8±3.3</td>
<td>55%</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>No</td>
<td>9</td>
<td>-</td>
<td>immuno</td>
<td>15.8±3.5</td>
<td>-</td>
<td>-</td>
<td>5.3</td>
<td>-</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes</td>
<td>20</td>
<td>-</td>
<td>LC-MS</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>4.8</td>
<td>-</td>
</tr>
<tr>
<td>Ireland</td>
<td>Voluntary</td>
<td>Yes</td>
<td>23</td>
<td>-</td>
<td>LC-MS</td>
<td>23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UK</td>
<td>Voluntary</td>
<td>Yes (39%)</td>
<td>29</td>
<td>immuno</td>
<td>LC-MS</td>
<td>29</td>
<td>-</td>
<td>-</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>Germany</td>
<td>Voluntary</td>
<td>Yes (90-93%, 83%≥1000 µg/d)</td>
<td>364</td>
<td>immuno</td>
<td>LC-MS</td>
<td>364</td>
<td>-</td>
<td>-</td>
<td>125</td>
<td>-</td>
</tr>
<tr>
<td>Canada</td>
<td>Limited</td>
<td>Yes</td>
<td>125</td>
<td>MBA</td>
<td>LC-MS</td>
<td>1900</td>
<td>-</td>
<td>-</td>
<td>50.6</td>
<td>-</td>
</tr>
<tr>
<td>Ireland</td>
<td>Mandatory</td>
<td>Yes</td>
<td>-</td>
<td>MBA</td>
<td>LC-tandem MS</td>
<td>5718</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Molloy et al. reported median(IQR) unless otherwise stated. \textsuperscript{a}Obeid et al.2005 reported geometric mean±SD. \textsuperscript{b}Fryer et al. reported mean±SD. \textsuperscript{c}Molloy et al. reported median(10\textsuperscript{th}-90\textsuperscript{th} percentile). \textsuperscript{d}Obeid et al. reported median(10\textsuperscript{th}-90\textsuperscript{th} percentile). \textsuperscript{e}Pentieve et al 2016. Reported mean±STD of placebo group. Immuno, protein-binding immunoassay; MBA: microbiological assay, HPLC: high performance liquid chromatography, LC-MS: liquid chromatography-mass spectrometry. Table modified from Plumptre 2016 thesis\textsuperscript{33}. 

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Given folate’s role in cellular development, it is not surprising to find a high demand for folate.

Furthermore, previous studies have found and confirmed higher circulating folate concentrations in cord compared to maternal blood. In the past fifteen years, research has focused on low maternal intake of folate to decrease risk of neural tube defects (NTDs) in their offspring (See Section 2.1.6.1). However, recently, due to the drastic increase in intakes, and serum and blood concentrations of folate resulting from mandatory folic acid fortification in the population among many countries; as well as an increase in periconceptional folic acid supplementation, a large body of research has shifted its focus to health effects and disease risk of excessive intakes of folate. In addition, this study has assessed the literature and ranked the strength of evidence as ‘convincing’, ‘probable’ or ‘possible/insufficient’ (Table 2.1.6.4).

### Table 2.1.6.4. Protective and adverse effects of periconceptional folic acid supplementation on pregnancy and birth outcomes

<table>
<thead>
<tr>
<th>I. Pregnancy &amp; birth outcomes</th>
<th>Protective effects</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convincing</td>
<td>• NTDs</td>
<td></td>
</tr>
<tr>
<td>Probable</td>
<td>• Congenital heart defects</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Preeclampsia</td>
<td></td>
</tr>
<tr>
<td>Possible/Insufficient</td>
<td>• Preterm birth</td>
<td>• Small for gestational age babies (with low maternal vitamin B₁₂)</td>
</tr>
<tr>
<td></td>
<td>• Low birth weight</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Infant health outcomes</th>
<th>Protective effects</th>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convincing</td>
<td>• Pediatric cancers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Language development</td>
<td></td>
</tr>
<tr>
<td>Probable</td>
<td>• Autistic traits</td>
<td>• Asthma/wheezing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Adolescent adiposity and insulin resistance</td>
</tr>
</tbody>
</table>
I. Pregnancy & Birth Outcomes

Convincing

Neural Tube Defects

NTDs refer to a complex of multifactorial disorders where the brain and spinal cord of the developing fetus fail to properly develop. This occurs during early embryogenesis, between conception and approximately 21-28 days. The evidence linking folate deficiency with NTDs has been strong and consistent. The first observed link was observed in the Leeds Pregnancy Nutrition Study in 1969. Two decades later, two large randomized double-blind clinical trials demonstrated the protective effect of periconceptional folic acid supplementation on NTD risk in women. The Medical Research Council Vitamin Study Research Group clinical trial involving 1195 women across seven countries found a 72% decreased risk of having a NTD-affected pregnancy with 4000 µg folic acid periconceptionally among women who previously had an NTD-affected pregnancy. Another clinical trial (n=4753) conducted as part of the Hungarian Family Program in 1984 reported a 27% reduction in the first NTD occurrence in response to 800 µg/d of folic acid as part of a multivitamin supplement consumed during the periconceptional period.

Probable

Preeclampsia

Preeclampsia is characterised by hypertension and proteinuria during pregnancy. Few studies have associated periconceptional use of folic acid with a decreased risk of preeclampsia. Earlier studies reported a 43-63% decrease in preeclampsia associated with multivitamin supplements containing folic acid. However, no specific micronutrient was attributed to this outcome. Furthermore, some studies did not find a significant association between folic acid supplementation in the range of 400-500 µg and preeclampsia. Nevertheless, more recent studies conducted in China and Canada found a dose-response inverse relationship between folic acid supplementation and preeclampsia. In the Canadian
study, the protective effect of folic acid supplementation was more pronounced for mothers with a higher risk of developing preeclampsia\textsuperscript{151}. Although this dose-response was not seen in a US study, folic acid supplementation in early pregnancy was associated with a protective effect on preeclampsia on lean compared to obese mothers\textsuperscript{154}.

**Congenital Heart and Other Congenital Defects**

Both clinical\textsuperscript{155} and observational studies in the US\textsuperscript{156,157}, Netherlands\textsuperscript{158} and China\textsuperscript{159} have generally shown an inverse association between maternal folic acid supplementation and congenital heart defects. The most recent population-based case-control study found that approximately 40\% of congenital heart defects may be preventable with folic acid supplementation of 600 µg/d\textsuperscript{160}. Although consistent and unequivocal evidence for the protective effect of periconceptional folic acid supplementation on NTD risk exists, evidence for protective effects of folic acid supplementation on other congenital defects have not been well established\textsuperscript{161}. This may be due to varying folate status of the populations studied\textsuperscript{166}.

**Possible/Insufficient**

**Preterm Birth**

Most studies did not find significant associations between folic acid supplementation and preterm birth\textsuperscript{154,162,163}. However, some studies have reported that a higher plasma folate concentration during late pregnancy is associated with longer gestation and lower risk for preterm births\textsuperscript{164,165}. Furthermore, a recent study conducted in China found an 8\% lower risk of preterm birth with periconceptional folic acid use\textsuperscript{152}.

**Low Birth Weight and Small for Gestational Age (SGA)**

Among the previously mentioned pregnancy outcomes, low birth weight and SGA are considered major influencers of infant long term health\textsuperscript{166}. Numerous studies have reported associations between one-carbon
nutrients during pregnancy and the risk for low birth weight and SGA newborns. A study in Greece reported that supplementation with high folic acid (5000 µg/d) in early to mid-pregnancy was associated with a 60% decrease in the risk of a low birth weight newborn and a 66% decrease in risk of a SGA newborn\textsuperscript{165}. Studies in the Netherlands and Pakistan also found that low maternal folate concentrations were associated with lower birth weight, increased risk for SGA\textsuperscript{167}, and intrauterine growth restriction\textsuperscript{168}. More recently, a study in China found a 19% decreased risk of SGA with 400 µg/d of folic acid\textsuperscript{152}. However, other studies did not find significant associations between maternal folate status and SGA\textsuperscript{154,164} or low birth weight\textsuperscript{154}. Nevertheless, a review of observational studies reported that maternal folate status has been positively associated with fetal growth parameters\textsuperscript{169}. The most consistent positive association was observed for RBC folate concentration and fetal growth parameters\textsuperscript{169}. Comparably, animal studies have shown positive associations with maternal methyl-supplemented diets and offspring birth weight. A study in rats fed a low-methionine diet induced higher homocysteine concentrations and lower fetal and maternal weight\textsuperscript{170}. Another study that fed rats a methyl-supplemented (including folic acid) diet reported decreased offspring leptin concentrations and impaired post-natal growth in both sexes\textsuperscript{171}. Increased long-term body weight gain was observed in males\textsuperscript{171}. Similar increases in body weight gain and impaired fetal development was observed in mice with combined fetal exposure to arsenic and maternal folic acid supplementation\textsuperscript{172}.

II. Infant Health Outcomes

Probable

Pediatric Cancers

A number of studies have associated periconceptional folic acid supplementation with a reduction in risk of developing pediatric cancers. An Australian case-control study associated folic acid supplementation with a decreased risk of childhood brain tumours\textsuperscript{171}. Similarly, as part of the Childhood Leukemia International Consortium, 12 case-control studies were analysed in order to investigate the association between prenatal...
use of folic acid and acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). This study found a reduced risk of ALL associated with maternal folic acid supplementation regardless of period of intervention (preconception or during pregnancy); however, only a weak inverse association was reported between prenatal folic acid use\textsuperscript{174} and AML. Another meta-analysis found a reduction in the risk of pediatric leukemia, brain tumours and neuroblastoma associated with the use of multivitamins containing folic acid; however, the study could not attribute the protective effective to a specific vitamin(s)\textsuperscript{175}. In contrast, some studies have reported that periconceptional folic acid supplementation may be associated with an increased risk of \textsuperscript{176,177} or a null\textsuperscript{178} effect on risk of pediatric cancers. Animal studies have shown that maternal folic acid supplementation decreases colon cancer risk\textsuperscript{179}, while it increases the risk of mammary tumours\textsuperscript{180} in the offspring. However, no human studies have examined the effect of periconceptional folic acid supplementation on colon and breast cancer risk in the offspring.

Language Development

Several studies have investigated a possible link between language development and periconceptional folic acid supplementation and have generally found an inverse association. The Rhea Study in Greece reported improvements in vocabulary, communication skills and verbal comprehension in 18-month-old children associated with high (≥5000 µg/d) folic acid supplementation during pregnancy\textsuperscript{181}. Similarly, a Norwegian Mother and Child Cohort Study (MoBA) found that folic acid supplementation from preconception to eight-week gestation decreased the incidence of severe language delay in children at 3 years of age\textsuperscript{182}.

Possible/Insufficient

Autistic Traits

Studies investigating the relationship between autistic spectrum disorders or autistic-behaviours and maternal folic acid supplementation have reported equivocal findings. The MoBA study in Norway found a
protective effect on autism spectrum disorders in 3-year-old children born to mothers who were supplemented with folic acid for 4-8 weeks preconception compared with those born to mothers who did not receive preconceptional folic acid supplementation. Other studies have also provided evidence that prenatal folic acid use is associated with decreased childhood autistic-behaviours, although other studies have reputed the purported protective effect of maternal folic acid supplementation on autistic traits.

Asthma/ Wheeze

The relationship between maternal folic acid supplementation and respiratory health in the offspring (i.e. asthma and wheezing) has been studied with conflicting results. Two meta-analysis of 14 studies (10 cohorts, 3 nested case-controls, and 1 case-control) and another more recent analysis looking at 10 large prospective cohorts did not find an association between childhood asthma and prenatal folic acid supplementation. However, another meta-analysis of 26 studies (16 cohorts, 7 case-controls, and 3 cross-sectional) found that early pregnancy folic acid supplementation was associated with an increase in the risk of wheeze. Interestingly, irrespective of folic acid supplementation, children homozygous for the TT genotype of the MTHFR677T polymorphism showed a higher risk for developing asthma in this meta-analysis. Other studies indicate that folic acid supplementation later in pregnancy (30-34 weeks gestation) may be protective against asthma in children at three and a half years of age.
See II. Excess Folate, Insulin Resistance and Adiposity in Section 2.1.6.1.

In summary, high prenatal supplementation of folic acid and folate status has been shown to have both protective and adverse effects on mothers and their offspring. Although the evidence may still be equivocal for the most part, the risk of high folic acid intakes (mostly from supplemental sources) and especially in combination with low $B_{12}$, may be potentially harmful to offspring’s health. Therefore, more research is needed to elucidate the effect of periconceptional folate status and supplementation on pregnancy and birth outcomes and infant health.
2.2 Vitamin B\textsubscript{12}

2.2.1 Definition, Chemical Structure and Dietary Sources

Vitamin B\textsubscript{12} (cobalamin) is a water-soluble B vitamin that is essential for RBC production, normal neurological function and metabolism of carbohydrates to glucose\textsuperscript{14}. There are several different B\textsubscript{12} forms depending on the moiety attached to cobalt in the centre of this molecule; including methylcobalamin, cyanocobalamin, hydroxycobalamin, sulfocobalamin and 5’-deoxyadenosylcobalamin (Figure 2.2.1). The two metabolically active forms are methylcobalamin and deoxyadenosylcobalamin\textsuperscript{95}. Cyanocobalamin is the most stable form and is found in supplements and fortified foods. Cyanocobalamin is also the form that is readily converted to methylcobalamin and 5’-deoxyadenosynylcobalamin, where methylcobalamin is a coenzyme for methionine synthase and 5’-deoxyadenosynylcobalamin is a coenzyme for amino and fatty acid metab.

![Chemical structures of vitamin B\textsubscript{12}](image)

**Figure 2.2.1. Chemical structures of vitamin B\textsubscript{12}.** These partial structures of B\textsubscript{12} indicate the changes in the random group (L) where; (1), 5’-deoxyadenosylcobalamin; (2), hydroxycobalamin; (3), methylcobalamin; (4), sulfocobalamin; (5), cyanocobalamin. Reprinted with permission from Watanabe 2007\textsuperscript{190}.

Animal sources are major sources of dietary B\textsubscript{12}; plants generally don’t provide much if any B12 except edible algae. Main animal sources are meats, eggs, fish and shellfish\textsuperscript{14}. Some examples of fortified foods with B\textsubscript{12} generally involve meat replacements such as soy and tofu or plant-based beverages\textsuperscript{190}.
2.2.2 Absorption, Transport and Storage

Before absorption of dietary B\textsubscript{12}, in the small intestine, B\textsubscript{12} must first be released from the protein it is bound to in food via hydrochloric acid and pepsin in the stomach. B\textsubscript{12} present in fortified foods, however, is unbound and can be absorbed immediately. Free B\textsubscript{12} attaches to R proteins called haptocorrins (produced in the salivary glands) to aid transport to the duodenum. In the duodenum, pancreatic proteases cleave and release B\textsubscript{12} bound to haptocorrins, which then bind to intrinsic factor (IF), a glycoprotein secreted by the parietal cells of the stomach forming the IF-B\textsubscript{12} complex. IF-B\textsubscript{12} travels to the terminal ileum and is absorbed by cubilin, cubam and amnionless receptors by calcium-dependent endocytosis (Figure 2.2.2).

These receptors can be saturated at 1.5 – 2.0 µg of B\textsubscript{12}, which limit absorption. The alternative pathway, whereby ~ 1% of B\textsubscript{12} is absorbed through passive diffusion, is relatively inefficient\textsuperscript{95}. Upon entry into the enterocyte, IF-B\textsubscript{12} complex dissociates and B\textsubscript{12} binds to plasma binding protein transcobalamin II. Upon exit into circulation, there are three plasma binding proteins (transcobalamin I, II or III) that bind B\textsubscript{12}. Transcobalamin I binds the majority (~80%) of circulating B\textsubscript{12} and stores it in the liver. Transcobalamin III has a similar binding role but this is still unclear. Transcobalamin II transports B\textsubscript{12} to the tissues through transcobalamin II receptor (TCII-R). Transport of B\textsubscript{12} involves two main transport proteins: haptocorrin and transcobalamin II. Most bind to haptocorrin and around 20-30% bind to transcobalamin II to form holotranscobalamin (holoTC)\textsuperscript{95} (Figure 2.2.2).
**Figure 2.2.2. B₁₂ absorption and transport.** P, dietary protein from food; HCl, hydrochloric acid; R; R proteins; IF, intrinsic factor; IF-B₁₂; intrinsic factor- B₁₂ complex; TC, transcobalamin; and HC, haptocorrin. Reprinted from Hughes et al. 2013⁹⁵.

HoloTC is the metabolically active fraction of B₁₂ as it represents the fraction of circulating B₁₂ for cellular uptake¹¹,⁹⁵. The body stores around 500 µg B₁₂ in the liver and excretes 1 – 10 µg B₁₂/d into bile, where 90% is reabsorbed and 10% is excreted in feces. For this reason, B₁₂ deficiency intakes years to develop in healthy individuals. Nevertheless, B₁₂ deficiency can be caused by either inadequate intake and/or impaired absorption (Table 2.2.2). Healthy adults with normal digestive systems are believed to absorb 50% of dietary B₁₂¹⁹⁰. However, gastrointestinal function is believed to decrease with age and is suggested to be due to gastrointestinal atrophy and reduced gastric acidity. Malabsorption of B₁₂ can be attributed to the lack of IF due to loss of gastric parietal cells, resulting in pernicious anemia (Table 2.2.2). Hence, a higher prevalence of B₁₂ deficiency is seen in the elderly¹⁴,⁶⁹,⁹⁵. Nevertheless, in light of this, older adults have been shown to increase their supplementation of B₁₂, especially in older women. Hence, despite the possibility of decreased intakes of animal products and malabsorption, no significant differences were observed in B₁₂ deficiency across the lifecycle⁶⁹.
Table 2.2.2. Causes of B<sub>12</sub> deficiency

<table>
<thead>
<tr>
<th>Cause</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary inadequacy</td>
<td>Diet low in meat and dairy products</td>
</tr>
<tr>
<td></td>
<td>Alcohol consumption</td>
</tr>
<tr>
<td>Malabsorption</td>
<td>Lack of IF</td>
</tr>
<tr>
<td></td>
<td>Pancreatic insufficiency</td>
</tr>
<tr>
<td></td>
<td>Parasitism</td>
</tr>
<tr>
<td></td>
<td>Transcobalamin deficiency</td>
</tr>
<tr>
<td></td>
<td>Gastric bypass or other bariatric surgery</td>
</tr>
</tbody>
</table>

2.2.3 Biochemical Functions

The two metabolically active forms of vitamin B<sub>12</sub> are methylcobalamin and 5’-deoxyadenosylcobalamin. Methylcobalamin serves as a coenzyme for MS in the one-carbon metabolism pathway. Methylcobalamin with MS transfers a single methyl from 5-MTHF to homocysteine to form methionine and regenerate THF. This process is important in combination with folate/folic acid as THF can be converted to 5, 10-methylene THF via B<sub>6</sub>-dependent SHMT for de novo DNA synthesis (Figure 2.2.3a).

Figure 2.2.3a. Biochemical functions of B<sub>12</sub> (methylcobalamin) in folate/folic acid dependent one-carbon metabolism. Enzymes are shown in bold. MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; DMG, dimethylglycine; Met, methionine; MTHFR, methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; tHcy, homocysteine; THF, tetrahydrofolate. Reprinted with permission from Masih 2013<sup>191</sup>.
5'-deoxyadenosylcobalamin serves as the coenzyme for L-methylmalonyl-CoA mutase, which is the enzyme that catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA. This enzymatic reaction is involved in amino and fatty acid metabolism\(^\text{190}\) (**Figure 2.2.3b**).

**Figure 2.2.3b.** Biochemical functions of B\(_\text{12}\) (5’deoxyadenosylcobalamin) in amino and fatty acid metabolism. Enzymes are shown in bold. MCM, methylmalonyl-CoA mutase; MMA, methylmalonic acid. Reprinted with permission from Masih 2013\(^\text{191}\).

2.2.4 Vitamin B\(_\text{12}\) Intake Requirements

For healthy adults between 19 – 50 years old, the EAR and RDA are set at 2.0 and 2.4 µg/d. The EAR is based on the minimum serum B\(_\text{12}\) concentration needed to prevent adverse hematological effects. Not enough information is available to determine the RDA; hence it is set by assuming the RDA is equal to the EAR plus twice the coefficient of variation of 10% of the standard deviation of the B\(_\text{12}\) requirement. Therefore, the RDA is approximately 120% of the EAR. The EAR and RDA are increased for pregnant women to 2.2 and 2.6 µg/d, respectively. This increase in requirement is suggested to be due to an estimated B\(_\text{12}\) fetal deposition of 0.1 to 0.2 µg/d. For lactating women, due to a loss of approximately 0.25 - 0.33 µg/d of B\(_\text{12}\) in milk, the EAR and RDA are increased to 2.4 to 2.8 µg/d. A UL has not determined as current evidence suggest no adverse effects associated with excess B\(_\text{12}\)\(^\text{14}\) (**Table 2.2.4**).
Table 2.2.4. 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>2.0</td>
<td>2.4</td>
<td>ND</td>
</tr>
<tr>
<td>Female, pregnant</td>
<td>2.2</td>
<td>2.6</td>
<td>ND</td>
</tr>
<tr>
<td>Female, lactating</td>
<td>2.4</td>
<td>2.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

DFE, dietary folate equivalents; EAR, estimated average requirement; RDA, recommended dietary allowance; UL, tolerable upper limit; ND, not determined.

Data from the US generally report an adequate intake of B₁₂ in the general population. In the 2003-2006 NHANES data, adult women consumed a median of 3.4 µg/d from a diet containing enriched cereal grains¹⁹². However, when accounting for both enriched cereal grains and other supplemental sources, the median increased to 16.5 µg/d¹⁹². Unlike in the US, intakes in Canada are reported to be less adequate with up to 20% of females (≥14 years old) consuming dietary B₁₂ (natural and fortified sources) at less than the EAR¹⁹³. However, inadequacy decreased to <5% when accounting for supplemental intakes.

2.2.5 Biomarkers of Vitamin B₁₂

There are four main biomarkers to measure B₁₂ status; plasma/serum B₁₂, holoTC, methylmalonic acid (MMA) and homocysteine (Table 2.2.5). Serum B₁₂ is primarily used as an assessment of clinical status as it measures circulating B₁₂. Similarly, holoTC measures the concentration of B₁₂ available for cellular uptake. MMA and homocysteine are inverse functional indicators of B₁₂ status. With low B₁₂, B₁₂ (5’deoxyadenosylcobalamin)-dependent MCM activity decreases, and thus increases conversion of L-methylmalonyl-CoA to MMA via D-methylmalonyl-CoA hydrolase (Figure 2.2.3b). Homocysteine is increased with low B₁₂ as MS activity is reduced; however, homocysteine is a less specific inverse functional indicator to B₁₂ as it is influenced by other B-vitamins such as B₂, B₆ and folate.

Cutoffs for deficiency have not unequivocally been established as a low serum B₁₂ concentration does not necessarily equate to deficiency, and similarly, a level above a ‘normal’ cutoff does not equate to
adequacy\textsuperscript{130}. Most laboratories generally use a reference range to determine its own deficiency range depending on the assay method. Over the past thirty years, the gold standard method has shifted to the protein binding assay over the microbiologic assay\textsuperscript{27,95}. A serum B\textsubscript{12} concentration of 150 pmol/L is commonly considered a deficiency cutoff\textsuperscript{11}, 150 to 258 pmol/L for a marginal or subclinical\textsuperscript{194,195}, and 220 pmol/L for maximal protection against NTDs\textsuperscript{196}. As there is no set adequacy cutoff for serum B\textsubscript{12}, a value above deficiency has been more commonly used\textsuperscript{130,197,198}. For MMA, a cutoff >271 nmol/L is considered a deficiency cutoff\textsuperscript{68}. For homocysteine, a cutoff >13 µmol/L could indicate B\textsubscript{12} deficiency\textsuperscript{69,199}. For holoTC, deficiency is considered to be <35 pmol/L\textsuperscript{137}.

For more accurate assessment of B\textsubscript{12} status, the NHANES roundtable summary suggested the need to measure and report a combination of at least one biomarker of circulating B\textsubscript{12} (serum B\textsubscript{12} or holoTC) and one functional biomarker (MMA or homocysteine)\textsuperscript{11}.

| Table 2.2.5. Comparison of B\textsubscript{12} status biomarkers |
|---|---|
| Biomarker | Type of biomarker | Strengths | Weaknesses |
| Plasma/serum B\textsubscript{12} | Circulating measure | - Measures total circulating vitamin B\textsubscript{12}  
- Indicative of long-term low B\textsubscript{12} intake | Needs confirmation with MMA or homocysteine |
| Holotranscobalamin (holoTC) | Circulating measure | - Measures metabolically active portion of vitamin B\textsubscript{12}  
- Sensitive to B\textsubscript{12} deficiency status | Ongoing studies; holoTC concentrations are understudied |
| Methylmalonic acid (MMA) | Functional marker | - Elevated MMA is more specific indicator for B\textsubscript{12} deficiency than homocysteine  
- Sensitive to B\textsubscript{12} deficiency status | MMA not a good functional marker of B\textsubscript{12} status in certain cases (e.g. pregnancy) |
| Plasma homocysteine | Functional marker | - Elevated homocysteine can be indicator B\textsubscript{12} deficiency | Influenced by other B-vitamins (folate, B\textsubscript{2} and B\textsubscript{6}) |

Table modified from Plumptre et al. 2016 thesis\textsuperscript{33}.
2.2.6 Vitamin B\textsubscript{12} and Health

2.2.6.1 Vitamin B\textsubscript{12} in Health and Disease

B\textsubscript{12} deficiency has been linked to neurological symptoms and sensory neuropathy\textsuperscript{14}. These manifestations include: megaloblastic anemia, myelopathy, neurodegeneration, depression and cognitive decline\textsuperscript{14,69,200}. Although B\textsubscript{12} deficiency can occur without masking by folate, they are integrally linked. High folic acid intake has been associated with the masking of B\textsubscript{12} deficiency through the methyl trap and has been shown to mask hematological manifestations, thereby delaying the diagnosis of B\textsubscript{12} deficiency. Interestingly, a study reported that the proportion of individuals with low B\textsubscript{12} without macrocytosis (undiagnosed B\textsubscript{12} deficiency) was higher in the post- than in the pre-folic acid fortification period\textsuperscript{201}. Furthermore, while the requirements for folate have been met by fortification and supplementation practices, the same cannot be said for B\textsubscript{12} as it remains untreated and inadequate (<111 pmol/L) among 35% of women of reproductive age\textsuperscript{202}.

2.2.6.2 Vitamin B\textsubscript{12} in Pregnancy

I. Maternal B\textsubscript{12} Intakes and Low B\textsubscript{12} Levels

Low maternal concentrations of B\textsubscript{12} have been reported despite adequate intakes. Most prenatal supplements contain the amount of B\textsubscript{12} equal to the RDA (2.6 µg/d). The PREFORM study found that greater than 90% of women had intakes that met the RDA\textsuperscript{115}. Serum holoTC concentrations were found to be in the normal range at 35 to 140 pmol/L in 88 to 91% of pregnant Canadian women from the Alberta Pregnancy Outcomes in Nutrition (APrON)\textsuperscript{137}. Nevertheless, the population data based on the 2007-2009 CHMS reported approximately 6% and 20% of the Canadian women between 20 to 79 years old with serum B\textsubscript{12} indicative of deficiency (<148 pmol/L) and marginal status (148-220 pmol/L), respectively\textsuperscript{69}. A study in the Netherlands showed a progressive decline reaching marginal and deficient (<150 pmol/L) serum B\textsubscript{12} by 28 – 32 weeks gestation\textsuperscript{201}, suggesting that B\textsubscript{12} concentrations decrease as pregnancy progresses. Similar findings were reported by more recent studies from Canada. In the PREFORM study,
17% and 38% of women in early pregnancy and at delivery, respectively, had deficient (<138 pmol/L) serum B₁₂ concentrations. A study from Vancouver found 23 and 35% of women had deficient (<148 pmol/L) or marginal (148-220 pmol/L) plasma B₁₂ concentrations, respectively, in late pregnancy. Newborn B₁₂ concentrations have been shown to be significantly higher than maternal concentrations. A study reported cord serum B₁₂ concentration (268 pmol/L) to be double that in maternal serum (188 pmol/L). Another study reported similar findings with serum B₁₂ in cord blood as 321 pmol/L and in maternal blood at delivery as 169 pmol/L. At 27 weeks postpartum, the PREFORM study reported that infants had a serum B₁₂ concentration of 226 pmol/L where maternal B₁₂ concentrations at 20 and 36 weeks gestation were 177 pmol/L and 149 pmol/L, respectively. Other studies have also reported decreasing B₁₂ concentrations as pregnancy progresses despite adequate intakes (Table 2.2.6.2a). Animal studies show that B₁₂ accumulates in the placenta of pregnant rats injected with B₁₂ and that it is actively transported to fetal tissues in a dose-dependent manner. This implies that cord blood and maternal B₁₂ concentrations are closely correlated over the course of pregnancy. Hence, based on these studies, maternal B₁₂ status is important to ensure sufficient supply of B₁₂ is transferred to the fetus.

Reasons for this B₁₂ reduction in the pregnant mother has been attributed primarily to physiological changes occurring during pregnancy as well as to increased demands to ensure fetal growth and development. In the mother, some of these changes are related to hemodilution, hormonal changes, decreased B₁₂ absorption, alterations in B₁₂ placental transport and binding protein activity. In the fetus, changes mainly occur in order to support rapid embryonic growth and development involving DNA and protein synthesis.
Table 2.2.6.2a. Summary of studies investigating $B_{12}$ intakes and serum concentrations.

<table>
<thead>
<tr>
<th>Location</th>
<th>Pregnant</th>
<th>Sample size</th>
<th>$B_{12}$ Intake (µg/d)</th>
<th>Serum $B_{12}$ (pmol/L)</th>
<th>Median(range)</th>
<th>HoloTC (pmol/L) (&lt;140pmol/L)</th>
<th>Median(range)</th>
<th>MMA (µmol/L)</th>
<th>Median(range)</th>
<th>$B_{12}$ deficiency prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baker et al. 2002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes</td>
<td>563</td>
<td>12</td>
<td>$≤28$ gestn: $385$</td>
<td>(86-446)</td>
<td>(81,94)</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>$1^{st}$: 10 $≤28$ gestn: $5.2$</td>
</tr>
<tr>
<td>Ray et al. 2008&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Yes</td>
<td>10,622</td>
<td>-</td>
<td>$&gt;28$ gestn: $249$</td>
<td>(73-412)</td>
<td>(17,34)</td>
<td>90</td>
<td>-</td>
<td>-</td>
<td>$2^{nd}$: 203 $&lt;13$ $&lt;13$</td>
</tr>
<tr>
<td>MacFarlane et al. 2011</td>
<td>No</td>
<td>5,600</td>
<td>-</td>
<td>&gt;148</td>
<td>(64-323)</td>
<td>(77,83)</td>
<td>364</td>
<td>-</td>
<td>-</td>
<td>$3^{rd}$: 168 $&lt;13$ $&lt;13$</td>
</tr>
<tr>
<td>Wu et al. 2013&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Yes</td>
<td>554</td>
<td>-</td>
<td>$≤28$ gestn: $126$</td>
<td>(277-293)</td>
<td>(103-133)</td>
<td>467</td>
<td>-</td>
<td>-</td>
<td>$≤28$ gestn: $5.2$ $&lt;13$</td>
</tr>
<tr>
<td>Fayyaz et al. 2014&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Yes</td>
<td>645</td>
<td>-</td>
<td>&gt;200</td>
<td>(244-255)</td>
<td>(130-144)</td>
<td>390</td>
<td>-</td>
<td>-</td>
<td>$&gt;28$ gestn: $10.1$ &lt;13</td>
</tr>
<tr>
<td>Herran et al. 2015&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Yes</td>
<td>9,523</td>
<td>-</td>
<td>$≤28$ gestn: $287±126$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$≤28$ gestn: $5.2$ $≤13$</td>
</tr>
<tr>
<td>Visentin et al. 2016&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Yes</td>
<td>368</td>
<td>-</td>
<td>&gt;220</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$&gt;28$ gestn: $10.1$ &lt;13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Baker at al. 2002 and Wu et al. 2013 reported mean±STD unless otherwise stated. <sup>b</sup>Ray et al.2008 reported $B_{12}$ deficiency as $<125$ pmol/L. <sup>c</sup>Fayyaz et al.2014 reported median 95% CI. <sup>d</sup>Herran et al. 2015 reported standard errors for serum $B_{12}$, 95% CI for $B_{12}$ deficiency, and used a cutoff $<148$ pmol/L. <sup>e</sup>Visentin et al. 2016 reported mean 95% CI. Serum $B_{12}$ concentrations were from supplement users. $B_{12}$ deficiency cutoff was $<148$ pmol/L and for marginal deficiency* 128-220pmol/L serum $B_{12}$. Gestn, gestation; EP, early pregnancy (1-12 weeks gestation); LP, late pregnancy (12-16 weeks gestation) D, delivery (28-42 weeks gestation); NP, nonpregnant women.
II. Pregnancy and birth outcomes

The effects of B₁₂ supplementation on health effects during pregnancy, birth and in the infant are shown in Table 2.2.6.2b. In addition, this study has assessed the literature and ranked the strength of evidence as ‘convincing’, ‘probable’ or ‘possible/insufficient’.

Table 2.2.6.2b. Adverse effects of periconceptional B₁₂ supplementation on pregnancy and birth and infant health outcomes.

<table>
<thead>
<tr>
<th>Adverse effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Pregnancy &amp; birth outcomes</td>
</tr>
<tr>
<td>Probable</td>
</tr>
<tr>
<td>• NTDs</td>
</tr>
<tr>
<td>• Preeclampsia</td>
</tr>
<tr>
<td>• Preterm birth</td>
</tr>
<tr>
<td>• Low birth weight</td>
</tr>
<tr>
<td>• Small for gestational age babies (with high maternal folate)</td>
</tr>
<tr>
<td>II. Infant health outcomes</td>
</tr>
<tr>
<td>Probable</td>
</tr>
<tr>
<td>• Cognitive development</td>
</tr>
<tr>
<td>Possible/Insufficient</td>
</tr>
<tr>
<td>• Adolescent adiposity &amp; insulin resistance</td>
</tr>
</tbody>
</table>

Probable

NTDs

The evidence linking maternal B₁₂ and NTDs are few but generally points to an inverse association 211.

However, some studies did not find associations with NTD affected pregnancy and maternal B₁₂ status 197,212.

More recent case-control studies have reported an increased odds ratio of 2.5 to 5.4 for having a NTD affected pregnancy associated with low serum B₁₂ concentrations 196,213–217.

Possible/Insufficient

Preeclampsia

Associations between maternal B₁₂ status and the risk of preeclampsia have been inconsistent due largely to variable B₁₂ biomarkers used in these studies. Several studies have shown higher homocysteine concentrations in women with preeclampsia than in normotensive women; however, serum B₁₂ concentrations showed no significant difference between the two groups 218–220.
**Preterm Birth**

High homocysteine concentrations, as a proxy of B₁₂ insufficiency, have been associated with an increased risk of preterm birth²²¹,²²². However, a case-control study in India reported that higher concentrations of B₁₂ was associated with preterm rather than term deliveries²²³.

**Low Birth Weight and Small for Gestational Age**

Studies investigating an association between maternal B₁₂ status and birth weight and gestational age have reported conflicting findings, depending on the location of the study population. Studies based in Western populations such as in Australia²²⁴ and Netherlands¹⁶⁷ reported no associations between maternal serum B₁₂ concentrations during pregnancy and adverse pregnancy and birth outcomes (premature birth, intrauterine growth restriction, small for gestational age and preeclampsia). In contrast, studies in Eastern populations such as in India²²⁵,²²⁶ found that low serum B₁₂ concentrations in the first and third trimesters and in cord blood were associated with lower birth weights²²⁶. A similar study from Pakistan found an increased risk of intrauterine growth restriction with lower maternal serum B₁₂²²⁵ and high maternal homocysteine concentrations¹⁶⁸.

With regards to both folate and B₁₂, a prospective analysis in Bangalore (n=1838) found that prenatal folic acid supplementation in combination with low B₁₂ intake was associated with an increased risk of SGA¹². In a subgroup analysis, an additional risk of SGA was observed in the presence of low B₁₂ and high folate intakes in the second trimester¹². A similar study in Pune, India found that increasing folate:B₁₂ ratio was correlated with an increase in maternal plasma homocysteine concentrations and with a decreased in birth weight, birth length, head circumference and chest circumference in the offspring²⁰². Although a recent study did not find a significant association between maternal B₁₂ status and the risk for SGA and low birth weight infants, an adjusted risk of fetal macrosomia was significantly higher in those born to mothers with the lowest serum B₁₂ and highest serum folate quartile than those born to mothers with the highest quartile.
of serum B<sub>12</sub> and lowest quartile of serum folate.<sup>277</sup> These mothers had no evidence of gestational diabetes.<sup>277</sup>

Two studies investigated the association between maternal homocysteine concentrations based on the fetal MTHFR C677T genotype and offspring birth weight. A French study found that the MTHFR 677TT genotype had lower birth weight and birth length than those with the CC or CT genotypes. These differences persisted until 1 year of age.<sup>228</sup> A more recent study found that the CT/TT genotypes rather than the wild-type CC predicted higher maternal homocysteine concentration and lower offspring birth weight. Since homocysteine is a functional inverse indicator of both folate and B<sub>12</sub> status, these data suggest that low folate and/or B<sub>12</sub> status may be associated with low birth weight.<sup>229</sup> Other studies have also found similar results in that high maternal homocysteine concentrations were associated with lower birth weight.<sup>167</sup> In a Japanese study, a 0.1 µmol/L increase in maternal homocysteine concentration during the third trimester was associated with 151 g lower infant birth weight.<sup>141</sup>

III. Infant health Outcomes

Probable

Cognitive Development

Low maternal B<sub>12</sub> status has been associated with an increased risk of impaired mental and social development in infants. Studies that have investigated this relationship are mainly from India or South Asia, as many women in these countries are highly susceptible to a low B<sub>12</sub> status, given their vegetarian dietary practice.<sup>11,210–214</sup> The most recent Indian study in 2012, in which 62% of women were B<sub>12</sub> inadequate (<150 pmol/L), reported a positive association between maternal B<sub>12</sub> status and motor and mental (psychomotor) development quotients in children at two years of age.<sup>231</sup> Similarly, decreased cognitive function as measured by decreased or slower performance in short-term memory and sustained attention
was observed in nine-year-old children born to mothers at the lowest decile of $B_{12}$ concentration (<77 pmol/L) in comparison to those born to mothers in the highest decile (>225 pmol/L).

Adolescent Adiposity & Insulin Resistance

As mentioned previously in Section 2.1.6.4, limited studies have investigated maternal $B_{12}$ status with the risk of adiposity and insulin resistance in children. In a study from the UK including 91 mother-infant pairs, low serum maternal $B_{12}$ concentrations (<191 ng/L) were inversely associated with offspring’s Homeostasis Model Assessment 2-Insulin Resistance (HOMA-IR) and triglycerides and positively associated with HDL-cholesterol after adjusting for age and BMI. After adjusting for multiple confounders, however, significance was lost for triglycerides and HOMA-IR. In the Randomized Control Trial of Low GI diet (ROLO) study, maternal intake of micronutrients, including vitamins D, E, and $B_{12}$, magnesium and selenium, was positively associated with neonatal anthropometry. Third trimester $B_{12}$ intake was positively associated with birth weight. At a follow up in infants at 6 months-of-age, maternal first trimester intakes of $B_{12}$ was positively associated with a risk of overweight/obese infants; a $B_{12}$ intake greater than 1 µg was associated with a 2.5 fold increased risk. Another study found that children from mothers deficient in $B_{12}$ (<148 pmol/L) during early pregnancy had 26.7% higher HOMA-IR at 6-8 years of age than those from mothers who were $B_{12}$ sufficient during early pregnancy. This study did not find any significant association of HOMA-IR in children with maternal folic acid or other micronutrient supplementation (iron, zinc and multiple micronutrient supplementation).

With regards to the combined effects of folate and $B_{12}$, the Pune Maternal Nutrition Study found that low maternal serum $B_{12}$ concentrations (<150 pmol/L) in combination with high RBC folate concentrations were associated with higher central adiposity and insulin resistance in infants and 6 year old children. Furthermore, mothers in the extremes (highest folate and lowest $B_{12}$ concentrations) were reported to have children with the most insulin resistant. A recent animal study that attempted to replicate
the Pune study found conflicting results\textsuperscript{239}. Female mice were fed one of three diets containing folic acid (2 mg/kg) with B\textsubscript{12} (50 µg/kg diet), high folic acid (10 mg/kg) with B\textsubscript{12}, or a high folic acid without B\textsubscript{12} starting 6 weeks before mating and lasting through during mating, pregnancy and weaning. Male offspring were weaned onto a control or western diet. Amongst the control-fed offspring, males born to dams with the high folic acid and B\textsubscript{12} were heavier, in contrast to the Pune study, and male offspring from the high folic acid without B\textsubscript{12} showed reduced insulin concentrations. Glucose tolerance and baseline glucose concentrations in the offspring fed control or western diets were not associated with any of the three maternal diets\textsuperscript{239}. Despite this contradictory finding, the intervention may have been too short to elicit the full effects of reduced insulin concentrations on the offspring from the dams fed a high folic acid without B\textsubscript{12} diet. This study also did not measure maternal RBC folate concentrations, making it difficult to assess how high folate concentrations were. Nevertheless, in support of other studies mentioned in Section 2.1.6.4, this animal study did find an association with maternal folic acid supplementation and increased weight gain in the offspring.

In summary, low maternal B\textsubscript{12} has been primarily associated with adverse health effects for children. Of interest to this study is the combined effect of maternal high folate and low B\textsubscript{12} status on adiposity and insulin resistance in the offspring.
2.3 Epigenetics

2.3.1 Definition

Epigenetics differs from genetics in that it refers to heritable changes in gene expression that are independent of changes in the DNA sequence itself. Most of these heritable epigenetic changes are established during cell differentiation and maintained even after cell division, allowing cells to have distinct identities and functions while containing the same genetic code. Types of epigenetic mechanisms are DNA methylation, covalent histone modification, chromatin remodelling and RNA interference. Histone modification, chromatin remodelling and DNA methylation involve the regulation i.e. coiling and uncoiling of the local structure of chromatin. Chromatin is DNA wrapped with histones, specifically an octamer of four core histone proteins (H3, H4, H2A and H2B), to form repeating units of nucleosomes. Nucleosomes function to package DNA within a cell, and can alter accessibility of regulatory DNA sequences to transcription factors and thus regulate gene expression. RNA interference includes non-coding microRNAs (miRNAs) that regulate gene expression by posttranscriptional gene silencing. Epigenetic mechanisms such as DNA methylation are generated and in parallel to histone modifications. Altogether, these epigenetic mechanisms interact to regulate the genome. Epigenetic changes are potentially reversible by dietary and pharmacologic manipulations.

2.3.2 DNA Methylation

DNA methylation generally refers to the methylation of specific cytosines of cytosine-guanine (CpG) sequences in the genome. DNA methylation also occurs at sites other than CpGs and are called non-CpG DNA methylation. DNA methylation is species-, tissue- and cell type-specific and plays an important role in gene expression, in the maintenance of DNA integrity and stability, and in chromatin conformation. Up to 80% of all CpG sites in mammalian DNA are methylated and most CpG methylation occurs in low density CpG areas such as in exons, noncoding regions and repeat DNA sites.
region and exon 1 of many transcribed human genes (Figure 2.3.2). The characteristics of a CpG island commonly include a minimum of 200 to 500 base pairs, an observed: expected CpG ratio >0.6 to 0.64, and a GC content at 50 to 55%\textsuperscript{249}. Genome-wide methylation refers to the total cytosine methylation in the genome whereas gene-specific methylation refers to DNA methylation at specific genes.

In most cases, CpG promoter methylation is an inverse determinant of gene expression\textsuperscript{207}. That is in normal cells, CpG islands are unmethylated and allow for transcription; while in disease states such as cancer, CpG island methylation leads to stable heritable transcriptional silencing or inactivation\textsuperscript{250} (Figure 2.3.2). DNA methylation occurring in non-CpG islands is relatively rare in most mammalian cell-types, however, it is most abundant in oocytes, pluripotent embryonic stem cells, and mature neurons\textsuperscript{250}. The function of mammalian non-CpG methylation remains unclear\textsuperscript{250}. Aberrant or dysregulation of DNA methylation can affect developmental and cellular processes such as embryonic development, genomic imprinting, transcription, X chromosome inactivation, chromosome structure, and stability\textsuperscript{251}. This in turn can contribute to the development and progression of chronic diseases\textsuperscript{252}, including asthma\textsuperscript{253}, cancer\textsuperscript{4,247,252}, cardiovascular disease\textsuperscript{254}, and metabolic disorders\textsuperscript{170,255}.

DNA methylation of cytosine (5-methylcytosine; 5mC) has been thought of as stable, irreversible epigenetic marks until the discovery ten-eleven translocation (TET) proteins that oxidize 5mC to 5-hydroxymethylcytosine (5-hmC), which is a marker for active DNA demethylation\textsuperscript{210}. 5-hmC has also been linked to regulating transcription by attracting or repelling specific DNA-binding proteins\textsuperscript{250}. This suggests that DNA methylation may be more susceptible to environmental factors than previously thought\textsuperscript{256}. Biological functions of 5-hmC are not fully understood, but some studies have indicated its importance in embryonic and germ cell development and brain cancer\textsuperscript{250}. 
Figure 2.3.2 CpG island and gene body methylation in normal and tumor cells. CpG island methylation occurs near the promoter region, exon 1 (box 1), and untranslated regions. Methylation (black filled circles) in the remaining area is referred to as gene body methylation, such as in exons, noncoding regions and repeat DNA sites. In normal cells, a) CpG sites near promoter regions are usually unmethylated and allow for transcription to occur and b) CpG low dense areas are usually methylated. In tumor cells, loss of methylation in CpG depleted regions and a gain of methylation in promoter regions of CpG islands are generally observed. Methylated sites are filled with black circles and unmethylated are white circles. Adapted from the publisher John Wiley and Sons.

2.3.2.1 Regulation of DNA Methylation

DNA methylation is a dynamic process between active DNA methylation, and active and/or passive DNA demethylation. CpG DNMT1, 3a, 3b using SAM are involved in active DNA methylation (Figure 2.1.3). DNMTs facilitate conversion of SAM to SAH, and are inhibited by an accumulation of SAH. For active demethylation, methyl DNA binding domain protein-2 (MBD2) removes methyl groups from 5mC residues, and can occur independently of DNA replication. Passive demethylation occurs when DNMT1 are inactive during the cell cycle following DNA replication, and thus sites can remain unmethylated in the newly synthesized DNA strand.

As mentioned previously, DNA methylation of promoter region CpG islands usually results in transcriptional silencing, although a there are a few exceptions. Transcription silencing involves
regulation by numerous proteins that bind and alter the conformation of chromatin. For instance, when CpG islands are hypermethylated, histone deacetylases (HDACs) suppress transcription in a complex with a transcriptional repressor, methyl-CpG binding protein-2 (MeCP2), and DNMTs. This complex blocks the transcriptional machinery from accessing the promoter region (Figure 2.3.2.1). In contrast, when CpG islands are un/hypomethylated, transcription can occur as the nucleosomes are in a transcriptionally active state due to histone acetyltransferases that maintain an open chromatin conformation.

Figure 2.3.2.1. DNA methylation regulation of transcription. Hypermethylated CpGs (red circles) attract protein complexes, including HDACs, MeCP2, and DNMTs to bind and inhibit transcription by altering chromatin conformation to a closed state. Hypomethylated CpGs allow for transcription to occur as chromatin is in an open conformation. Adapted with permission from Bird 2002.

2.3.3 Epigenetic Reprogramming

Epigenetic reprogramming during embryogenesis starts with active and passive genome-wide demethylation, respectively, of the paternal and maternal genomes. This is followed by de novo methylation soon after implantation. Demethylation takes place during fertilization at the zygote stage, and then in the primordial germ cell, which develop to become a sperm or oocyte. DNA methylation content is the lowest at the blastocyst stage (Figure 2.3.3). Establishment of de novo methylation patterns by DNMT3a and 3b do not require pre-existing methylation and are thus able to establish a new pattern; in contrast,
maintenance of methylation patterns depends on DNMT1 to identify hemimethylated sites to ensure replication of DNA methylation patterns\textsuperscript{264}. The new DNA methylation pattern is considered stable postnatally but it can be modified by factors such as aging, cancer or the environment including diet.

**Figure 2.3.3. Epigenetic programming.** After fertilization, active demethylation of the paternal and passive demethylation of the maternal genome occurs. 5mC content is lowest at the blastocyst stage. Formation of de novo methylation patterns rise after implantation. ESCs, embryonic stem cells; ICM, inner cell mass cells (forms the embryo); TE, trophoectoderm cells (forms the placenta). Modified from Plumptre 2016 thesis\textsuperscript{13}. Reprinted with permission from Wu and Zhang 2014\textsuperscript{250}.

2.3.4 Analysis of DNA Methylation

Epigenetic analyses have progressed through many years of innovation. Sequencing technology for DNA methylation analyses, previously restricted to specific loci, are now performed on a genome-wide scale at single-base-pair resolution\textsuperscript{249}. Similarly, for DNA methylation profiling, current techniques utilize microarray hybridization instead of two-dimensional gel electrophoresis\textsuperscript{249,265}.

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DNA methylation measurements can be attained either as a pattern of methylated target sequences along a section of DNA or as an average methylation level at a single genomic locus across many molecules. The complexity in measurement comes from the varying DNA methylation patterns associated with each cell type, DNA sample and location in the genome. Since hybridization techniques do not accurately distinguish between methylated and unmethylated cytosines, and DNA methyltransferases are not present during PCR, DNA methylation patterns are erased during amplification. Hence, pretreatments are required in order to reveal the presence or absence of the methyl group at cytosines of CpG sites. Once completed, amplification or hybridization can proceed. There are three primary methods for a methylation-dependent pretreatment of DNA: (1) endonuclease digestion, (2) affinity enrichment, and (3) bisulphite conversion. After this step, various analytical steps including probe hybridization and sequencing proceed in order to determine the locations and patterns of 5mC residues.

The first of three approaches for methylation-dependent pretreatment of DNA is sequence-specific restriction endonuclease, which works in a complex with DNMTs to methylate bases in the recognition site as protection from the restriction defense system. The first locus-specific DNA methylation analyses uses methylation-sensitive restriction endonucleases followed by gel electrophoresis and hybridization on Southern blots; a method that is still used today for some locus-specific studies. Also currently in use is PCR across the restriction site as an alternative subsequent step to methylation-sensitive restriction digestion. This is a very sensitive method despite some risk of false-positive results caused by incomplete digestion.

The second approach involves chromatin immunoprecipitation (ChIP), microarray hybridization (ChIP-chip) or next generation sequencing (ChIP-seq) powerful techniques used primarily for genome-wide studies of histone modifications and DNA methylation. These methods identify the location of DNA-binding proteins and epigenetic marks in the genome. Another method of affinity enrichment involves
either 5mC-specific antibodies (in the context of denatured DNA) or methyl-binding protein with an 
affinity for methylated native genomic DNA. This tool is particularly useful for DNA methylation profiling 
in complex genomes. Despite the advantages of this approach, affinity-based methods do not yield 
information on individual CpG dinucleotides and require substantial experimental or bioinformatics 
adjustment for the non-uniform distribution of CpG sites at different regions of the genome.

The third approach of treating denatured DNA with sodium bisulphite depends on the conversion of only 
unmethylated cytosines to uracil (read as thymine when sequenced), while keeping methylated cytosines 
intact. Illumina has adapted the bisulphite conversion method to their Infinium platforms (e.g. the Illumina 
Infinium MethylationEPIC array used in this study. Bisulphite conversion is followed by whole-genome 
amplification, then fragmentation, and hybridization of DNA samples to methylation-specific DNA 
oligomers that are linked to individual bead types. Each bead type matches to a specific CpG site and 
methylation state to provide the DNA methylation status of individual CpG sequences.

The choice of method for DNA methylation analysis is complex and highly dependent on various factors 
and needs of the study. For instance, the number of samples, quality and quantity of DNA, potential 
 sources of bias, desired coverage, and resolution are some considerations. Additionally, accounting for the 
type of organism being studied is important; an array-based analysis would require the presence of a 
suitable array for the species of interest, whereas, a sequence-based analysis would be applicable to all 
species as long as a reference genome exists.

2.3.5 Epigenetics and Health and Disease

The Developmental Origins of Health and Disease (DOHaD) is a theory that explains the fetal origins of 
adult disorders through the involvement of epigenetics. This theory proposes epigenetics as a possible 
mechanism to explain the association between in utero environmental factors and infant/adult disease
outcomes. It suggests that metabolic, hormonal and/or nutritional changes in the intrauterine environment cause epigenetic changes, either genome-wide or gene-specific DNA methylation patterns, which in turn influence disease susceptibility and health outcomes in the offspring later in life (Figure 2.3.5).

Figure 2.3.5. DOHaD. Environmental factors can influence hormonal, metabolic or epigenetic changes in the mother altering gene expression and stability, which may influence chronic disease of offspring later in life.

Early observational studies have supported the DOHaD hypothesis, an example being the Dutch Winter Famine Cohort where undernourished mothers produced offspring with increased risk of metabolic and cardiovascular disease in adulthood. The same Dutch cohort and the Swedish Overkalix Cohort have also demonstrated that health outcomes of the grandchildren can be affected, suggesting that the effects of maternal exposures persist transgenerationally.

A key component of the DOHaD is the predictive adaptive response (PAR) hypothesis, which highlights the concept of ‘matched environments’ between the pre and postnatal environments. If the developing fetus can predict and adapt to the postnatal environment based on cues from the prenatal environment, it will be better suited for the future environment. If, however, mismatch occurs, the risk of developing adverse effects later in life increases. Epigenetic processes have been suggested as an underpinning
mechanism of PAR\textsuperscript{274} and several studies have supported this hypothesis\textsuperscript{275,276}. For instance, a rodent study reported that an obesogenic phenotype was prevented in offspring who were fed the same high vitamin diet as their mothers' (10 times the recommended level or 20 mg folic acid/kg), as male offspring born to high folate-fed mothers and fed a normal (2 mg folic acid/kg) folate were shown to have the highest body weight gain\textsuperscript{275}. Another study, however, did not show support for the PAR hypothesis\textsuperscript{277}. Female offspring born to dams fed a high folate diet (20 mg folic acid/kg) had reduced body weight gain was associated with smaller femoral area. Matching the high folate dam diet with a high folate diet of these female pups did not correct for the reduced body weight gain of the high folate dam diet. Nevertheless, the mismatch in maternal and pup diet did result in reduced femoral peak load strength and stiffness\textsuperscript{277}. A possible reason for this discrepancy could be attributed to varying critical periods of growth and development (either in utero, postnatally, or both) of each cell, tissue type or organ\textsuperscript{278}.

2.3.6 One-Carbon Nutrients and DNA Methylation

Epigenetics has emerged as a critical mechanism through which diet can influence the genome. There has been a tremendous amount of interest in this area\textsuperscript{269,279,280} but more insight is still needed regarding the effects of diets on epigenetic control, regulation, gene function, and ultimately human health and disease.

The influence of intrauterine one-carbon nutrients such as folate, B\textsubscript{12}, B\textsubscript{6}, and choline on DNA methylation status and subsequent changes in phenotype and disease susceptibility later in life has garnered significant research interest. Among these one-carbon nutrients, folate and B\textsubscript{12} have been most extensively studied. The primary reason for this is a biological mechanistic link that ties one-carbon nutrients with DNA methylation, as illustrated in the folate and B\textsubscript{12} pathway shown in Figure 2.1.3. Key elements in this link include: SAM as the universal methyl donor in the body; MS as an enzyme that transfers a methyl group from 5-MTHF to homocysteine to produce methionine; and B\textsubscript{12} as a coenzyme to MS.
Given the involvement of folate and B\textsubscript{12} in the transfer of methyl groups, alterations in concentrations of these nutrients during pregnancy can potentially modulate the methyl donor ‘pool’ available for DNA methylation reactions. Evidence from animal and human studies are summarised in Sections 2.3.7 and 2.3.8.

2.3.7 Maternal One-Carbon Nutrients on DNA Methylation and Health Outcomes in Animal Offspring

2.3.7.1 One-Carbon Nutrients on DNA Methylation on Growth and Development Outcomes

Animal studies have provided evidence of how one-carbon nutrients \textit{in utero} may change DNA methylation patterns, and how the exposure to these nutrients potentially affects offspring’s disease outcomes related to cancer, metabolic syndrome, adiposity, insulin resistance and obesity (Table 2.3.7).

**General methyl donors**

One of the more well-known animal studies demonstrating the effect of maternal one-carbon nutrient (folate acid, B\textsubscript{12}, choline and betaine) intake on offspring phenotype was conducted in the \textit{Agouti}\textsuperscript{281,282}. Interestingly, CpG hypomethylation of the promoter region of \textit{Agouti} gene in this model resulted in maximal \textit{Agouti} expression producing a yellow coat and more obese and prone to adult-onset obesity, diabetes and tumorigenesis\textsuperscript{281,284}. In contrast, offspring of dams fed ample one-carbon nutrients has increased CpG methylation and showed a brown coat colour and were less obese and diabetic\textsuperscript{281,284}.

Another well-cited animal model in this area is the \textit{AxinFused} mouse model, where a similar methyl-rich diet fed to dams produced a proportion of offspring with the kinked tail phenotype in the \textit{AxinFused} mouse model. This outcome was associated with higher CpG methylation in the promoter region of the \textit{AxinFused} gene\textsuperscript{284}. The same group of investigators also found that a methyl donor-deficient diet administered to mice for 60 days post-weaning resulted in loss of imprinting in \textit{IGF2}, which plays an important role in growth and development. This was considered a permanent effect on \textit{IGF2} as the loss persisted 100 days after post-weaning even on a control diet containing methyl group donors\textsuperscript{284}. Other animal studies have also shown
that methyl donor status in early life affects DNA methylation\textsuperscript{1,285–287} in genes related to mitochondrial metabolism and phospholipid homeostasis, in PGC-1\textalpha (energy metabolism)\textsuperscript{288}, and in PTPN22 (immune signalling) and PPAR\alpha (cholesterol and lipid metabolism) in rodent models\textsuperscript{289}. Furthermore, methyl donor supplementation during gestation showed differentially expressed metabolic genes in the liver and muscle, as well as a change in IYD (related to thyroid metabolism) methylation patterns in the F2 generation of a pig model\textsuperscript{290}. The associated phenotypic effects included decreased back fat percentage, adipose tissue, and fat thickness and increased shoulder fat percentage in the F2 generation that was fed the methylating micronutrients\textsuperscript{290}.

**Folate**

More than any other one-carbon nutrient, a large number of studies have investigated the effects of maternal folic acid supplementation on changes in DNA methylation patterns and consequent phenotypic or other health and disease outcomes. In mice heterozygous for folate binding protein gene (Folbp1\textsuperscript{+/-}), daily gavage of 5-formylTHF during periconception until 15.5 weeks gestation significantly decreased genome-wide DNA methylation in the liver and brain of offspring\textsuperscript{291}. In rats, folic acid supplementation three-weeks prior to mating and through pregnancy and lactation decreased genome-wide DNA methylation by 25\% in the liver of pups in comparison to those from dams fed a control diet\textsuperscript{292}. However, genome-wide DNA hypomethylation was observed in placental tissue of hyperhomocysteinemic dams fed a diet deficient in folate\textsuperscript{293}. The same study found positive correlations between placental genome-wide DNA methylation and folate concentrations in the placenta, plasma and liver\textsuperscript{293}. However, not all studies found changes in genome-wide DNA methylation in pups in response to maternal folic acid supplementation or folate status. In a rodent study in the fetal liver maternal folate deficiency during pregnancy did not change maternal and offspring genome-wide DNA methylation\textsuperscript{170}. Nevertheless, a low-methionine maternal diet did induce higher homocysteine concentrations and lower fetal and maternal weight\textsuperscript{170}.
In animal models of tumorigenesis, maternal folic acid supplementation has been shown to modify DNA methylation and risk of cancer in the offspring. Maternal folic acid supplementation of dams with 5 mg/kg folic acid (equivalent to ~1000 µg folic acid/day in humans) in utero during pregnancy and lactation significantly decreased mammary genome-wide DNA methylation by 7%, and increased the risk of mammary tumors in the offspring. In a colorectal cancer animal model similar to the previous study by Ly et al. 2011, contrasting findings were observed as maternal folic acid supplementation at the same level and duration significantly increased colorectal genome-wide DNA methylation by 3%, and reduced the risk of developing colorectal tumors in the offspring. These findings suggest that the effect of maternal folic acid supplementation on cancer risk may be organ specific and may be mediated in part by modifications in genome-wide DNA methylation.

The effects of maternal diet, including folic acid supplementation, on fetal DNA methylation have also been demonstrated at the gene-specific level. Promoter DNA methylation of the PPARγ (regulator of adipocyte differentiation) and GR (cellular proliferation and differentiation) genes was decreased by 20% and 22.8%, respectively, while its protein expression increased in offspring from dams fed a protein restricted diet during pregnancy. Maternal folic acid supplementation prevented these changes. PPARγ has recently been found to upregulate genes encoding chromatin modification enzymes such as histone lysine methyltransferases that regulate transcription of adipogenesis. Histone H3 at lys9 (H3K4) in particular has been associated with resistance to obesity. CpG methylation in the promoter of the GR gene has been shown to be positively associated with increased adiposity and blood pressure in adulthood, and this effect was more pronounced in children born to mothers with the most unbalanced diets based on macronutrient intake in pregnancy. Another study found that maternal folic acid supplementation significantly decreased DNA methylation in the CpG sites of the promoter regions of the PPARγ and ERα (transcription factors involved in adipogenesis and glucose metabolism) genes, in exons 6 and 7 of the p53
gene (tumour suppressor), and in exon 15 of the APC gene (tumour suppressor) but not in p16 gene promoter (tumour suppressor) in the liver of rat pups\textsuperscript{297}. Increased maternal folic acid intake before and during pregnancy was also found to produce loss of imprinting for IGF2 and H19 (tumour suppressor) genes in human cord blood leukocytes, with more pronounced effects in males\textsuperscript{134}.

\textbf{B\textsubscript{12}}

Studies examining the effects of maternal B\textsubscript{12} status on genome-wide or gene-specific DNA methylation are limited and are usually in combination with folic acid\textsuperscript{299}. Similar to folate deficiency, B\textsubscript{12} deficiency can result in DNA hypomethylation as it is required for synthesis of methionine and SAM\textsuperscript{298}. The effect of maternal folic acid supplementation (4X the basal requirement) with or without B\textsubscript{12} on placental genome-wide DNA methylation was studied in rats\textsuperscript{299}. This study found a decrease in genome-wide DNA methylation associated with folic acid supplementation in the absence of B\textsubscript{12}, suggesting that the ratio of folic acid and B\textsubscript{12}, rather than the actual supplemental level, may have an important role in determining genome-wide DNA methylation patterns\textsuperscript{299}. Periconceptional folate and B\textsubscript{12} restriction was also investigated in a sheep model, where aberrant DNA methylation patterns were reported in 4\% of the 1400 CpG islands examined in the offspring in association with both maternal folate and B\textsubscript{12}. Specifically, 88\% of these CpG island sites were hypomethylated relative to the controls\textsuperscript{117}. This study also found that the offspring, most notably, males, born to mothers with folate and B\textsubscript{12} restriction were obese and insulin resistant, had elevated blood pressure, and exhibited altered DNA methylation at a number of sites in the genome associated with adiposity, insulin resistance, and increased immune response\textsuperscript{117}. Another study showed that rat pups born to mothers deficient in folate and B\textsubscript{12} during gestation and lactation were associated with decreased birth weight, increased central fat mass, liver steatosis, and myocardium hypertrophy\textsuperscript{300}. These manifestations were linked to decreased expression of SIRT1 (intracellular protein regulator) and PRMT1 (transfers methyl groups) and hypomethylation of PGC1-\textit{a} (transcriptional regulator of genes related to energy metabolism)\textsuperscript{300}.  

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In summary, animal studies provide evidence that DNA methylation can be modulated by intrauterine exposure to varying levels of one-carbon nutrients such as folate and B₁₂, with resultant changes in phenotype and other functional outcomes. However, the extrapolation of animal data to humans has several limitations. For instance, genes investigated in mice may not be present in humans such as with A¹⁰ and Axin fusion genes. Most animal models use inbred strains unlike human offspring that are genetically and epigenetically diverse. Furthermore, mouse models produce immature offspring where many of the developmental processes that occur in humans during pregnancy are postnatal events in rats and mice, and may as a result affect susceptibility of the animal offspring to the postnatal environment. Despite these limitations, animal studies contribute significant insights due to commonalities of some human diseases (diabetes, obesity, an cancer) to certain animal model systems.

Table 2.3.7. Summary of in utero and perinatal folate and B₁₂ nutrient supplementation on DNA methylation and related health and disease outcomes in the animal offspring

<table>
<thead>
<tr>
<th>Study</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>Waterland et al. 2003&lt;sup&gt;282&lt;/sup&gt;</td>
<td>Mice A¹⁰/a</td>
<td>1) 5 g choline, 5 g betaine, 5 mg folic acid, 0.5 mg B₁₂ 2) 2.5 g choline, 2.5 g betaine, 2.5 mg folic acid, 0.25 mg B₁₂</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>Waterland et al. 2006&lt;sup&gt;284&lt;/sup&gt;</td>
<td>Mice Axin&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1) 5 g choline, 5 g betaine, 5 mg folic acid, 0.5 mg B₁₂ 2) 2.5 g choline, 2.5 g betaine, 2.5 mg folic acid, 0.25 mg B₁₂</td>
<td>2 weeks prior to and throughout pregnancy and lactation</td>
<td>Tail tip</td>
<td>Liver</td>
<td>Increased CpG methylation (p=0.009) leading to less tail kinks (p&lt;0.0001) Increased CpG methylation (p=0.05) leading to less tail kinks (p&lt;0.0001)</td>
</tr>
<tr>
<td>Finnell et al. 2002&lt;sup&gt;291&lt;/sup&gt;</td>
<td>Mice Folbp&lt;sup&gt;1⁻/⁻&lt;/sup&gt;</td>
<td>25 mg/kg/d 5-formylTHF by gavage</td>
<td>2 weeks periconception to 15.5 wk gestation</td>
<td>Liver Brain</td>
<td>Genomic</td>
<td>~4-fold decrease (p&lt;0.05) ~2-fold decrease (p&lt;0.05)</td>
</tr>
<tr>
<td>Ly et al. 2011&lt;sup&gt;280&lt;/sup&gt;</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>Mammary Genomic</td>
<td>7% decrease (P = 0.03)</td>
<td></td>
</tr>
<tr>
<td>Study</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>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------</td>
<td>-----------------</td>
<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sie et al. 2011</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>Colorectum</td>
<td>Genomic</td>
<td>3% increase (p = 0.007)</td>
</tr>
<tr>
<td>Sie et al. 2013</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 p53 exons 6 and 7, in APC in exon 15 but not p16 (p&lt; 0.05)</td>
</tr>
</tbody>
</table>
| Kim et al. 2009  | Rats    | 1) 8 mg/kg folic acid  
2) 8 mg/kg folic acid & 0.3% Hcy  
3) 0.3% Hcy | 4 wks prior to day 20 of pregnancy | Placenta | Genomic        | r = 0.752 (p= 0.0003)  
r = 0.700 (p= 0.0012)  
r = 0.819 (p< 0.0001) |
| Lillycrop et al. | Mice    | 1) Control protein (180 g/kg protein plus 1 mg/kg folic acid)  
2) Restricted protein (90 g/kg casein plus 1 mg/kg folic acid)  
3) Restricted protein plus 5 mg/kg folic acid | Pregnancy | Liver   | Genomic        | PPARγ decreased by 20.6% (p=0.001)  
GR decreased by 22.8% (p=0.01) |
| 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 day 21 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                                                              |
| Kulkarni et al. 2011 | Rats | 8 mg/kg folic acid & B12 -/- vs. 2 mg/kg folic acid & B12 -/- | Pregnancy | Placenta | Genomic        | Decreased (P < 0.05)                                                  |
| Sinclair et al. 2007 | Sheep | B12 & folate deficient vs. control | 8 weeks prior to 6 days after conception Periconception | Liver   | 1400 CpG sites  | 4% of CpG sites had altered status (P < 0.001)                         |

Table modified from Plumptre et al. 2016 thesis."
2.3.8 Maternal Folate and B\textsubscript{12} status on DNA Methylation and Health Outcomes in Human Offspring

2.3.8.1 One-Carbon Nutrients on DNA Methylation

A limited number of human studies have investigated the effects of maternal folate and B\textsubscript{12} concentrations on DNA methylation patterns, but only a few looked at metabolic outcomes, in the offspring (Table 2.3.8). Focusing on studies that are based on maternal and cord blood samples, human studies have generally reported inconsistent findings.

**Folate**

The majority of observational and clinical studies have investigated the relationship between maternal folate exposures and DNA methylation of the offspring, and have shown equivocal findings. An observational study in the Netherlands, with voluntary folic acid food fortification, reported a 4.5% increase in DNA methylation in IGF2 DMR (differentially methylated region) derived from whole blood of 17-month old children whose mothers were supplemented with 400 µg/d folic acid compared with those born to mothers who were not folic acid supplemented\textsuperscript{302}. Independent of maternal folic acid supplementation status, an inverse association was observed between IGF2 DMR methylation and birth weight in the offspring\textsuperscript{302}. In contrast, the recent North American Newborn Epigenetic Study (NEST) found that DNA methylation levels at the IGF2 DMR were not significantly different between infants born to folic acid supplemented and non-supplemented mothers\textsuperscript{303}. However, the same investigators found significantly lower H19 DMR methylation in infants born to mothers supplemented with folic acid compared with those born to mothers not supplemented with folic acid\textsuperscript{303}. In the Generation R study in the Netherlands, a positive association between IGF2 DNA methylation and maternal folic acid supplementation after the first trimester (>12 weeks gestation), but not with preconceptional or first trimester (<12 weeks gestation) folic acid supplementation, has been reported\textsuperscript{304}. However, this study found no association between maternal folic acid supplementation and DNA methylation in 11 regions of seven genes (NR3C1, DRD4, 5-HTT, KCNQ1OT1, MTHFR, IGF2 DMR, and H19)\textsuperscript{304}. With similar
fortification practice as the Netherlands, a study in France did not find a correlation between the combination of folic acid supplementation with B₁₂ and B₆ intake before or during pregnancy and DNA methylation of the PLAGL1 DMR. However, an American mother-child cohort found maternal folic acid supplementation to be positively associated with PLAGL1 DNA methylation in cord leukocytes. In a study based in the United Kingdom, with similar mandatory folic acid food fortification as the US and Canada, they reported an inverse association between long interspersed nucleotide element-1 (LINE-1) DNA methylation and PEG3 methylation with maternal folic acid supplementation, only after the first trimester. Overall, the varying outcomes of these studies may be due to the timing of prenatal folic acid supplementation during the gestational periods or from the folate status of the population that vary depending on the country’s folic acid fortification practice.

B₁₂

Some population-based studies have suggested that maternal B₁₂ intake may have a stronger influence on DNA methylation of the offspring than folate, and are associated with infant growth and development. A cross-sectional study reported that methylation of the P2 promoter of the imprinted IGF2 gene was inversely associated with maternal serum B₁₂ but not with maternal or cord blood RBC folate concentrations. Increased maternal B₁₂ during pregnancy was associated with decreased genome-wide DNA methylation in newborns while increased B₁₂ in newborns was associated with reduced DNA methylation of IGFBP3 (involved in intrauterine growth) gene. Promoter regions of the SREBF1 and LDLR (two key regulators of cholesterol biosynthesis) genes were hypomethylated in mothers with B₁₂ deficiency, and as a result, the expression of downstream effector genes, including IDH1, SQLE, SC4MOL and INSIF1, STarD4, and cholesterol biosynthesis were significantly upregulated. Two US cohort studies found that the association between BMI and DNA methylated HIF3A consistently increased across tertiles of total B₁₂, folate, and B₆ intake, suggesting a potential causal relation between DNA methylation and adiposity through interactions with B₂, B₁₂ and folate total or supplemental intakes.
2.3.8.2 DNA Methylation on Health Outcomes

Several studies have investigated the effects of DNA methylation on offspring health outcomes with a specific focus on metabolic diseases (Table 2.3.8).

Three recent reviews investigating the epigenetic effects on obesity \(^{310-312}\) and type 2 diabetes \(^{311,312}\) have reported inconsistent associations between genome-wide DNA methylation and obesity and type 2 diabetes, although multiple obesity-associated differentially methylated sites in peripheral and cord blood cells were identified. Gene-specific DNA methylation studies have identified DNA alterations in various genes that are implicated in obesity, appetite control and metabolism, insulin signalling, immunity inflammation, growth and circadian regulation \(^{295,313,314}\). Studies have also examined measures related to metabolic syndrome and found candidate genes \(^{315-317}\) primarily associated with cortisol, adiposity and inflammation \(^{315}\) that are epigenetically modulated.

Genes related to BMI and body fat distribution have also been shown to be associated with changes in DNA methylation \(^{318-323}\). For instance, DNA hypermethylation of the \(HIF3A\) gene (related to neonatal and infant anthropometry) in blood and adipose tissue has been associated with greater infant weight and adiposity \(^{322}\).

In another study, 23 birth weight-associated CpG sites in 14 genes from cord blood were associated with developmental phenotypic characteristics in childhood \(^{324}\). However, beyond early childhood, the study found a lack of persistence in DNA methylation differences \(^{324}\). DNA methylation was also shown to affect genes related to the downstream effectors of \(PPARy\)-induced genes that determine mesenchymal stem cell differentiation into osteocytes and adipocytes, which may predispose the infant to fat accumulation \(^{325}\).

Similarly, studies have shown other measures such as waist-hip ratio \(^{326}\), blood lipids \(^{296,326,327}\), adiposity distribution at 5 years of age \(^{328}\) and at young adulthood \(^{329}\) associated with DNA methylation changes.
While some of the aforementioned studies reported significant changes in DNA methylation patterns as well as suggested possible functional outcomes in relation to birth weight, tumour development, obesity, metabolic syndrome, and other chronic diseases; the potential link between the observed DNA methylation changes and the aforementioned health outcomes have not been unequivocally established.

Furthermore, it is difficult to make clear conclusions regarding the associations between one-carbon nutrient-mediated epigenetic changes in utero and the risk of chronic disease later in life due to various factors that come into play. These factors include differences in study design, research models, techniques, and populations’ one-carbon nutrient status and/or supplementation habits.

**Table 2.3.8. Summary of in utero and perinatal folate and B₁₂ nutrient status on DNA methylation and potentially related health outcomes in human offspring**

<table>
<thead>
<tr>
<th>Study</th>
<th>One-carbon nutrient source &amp; levels</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect on DNA methylation</th>
<th>Outcomes on Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fryer et al. 2009³³⁰</td>
<td>Folic acid supplement, 400 µg/day</td>
<td>Pregnancy</td>
<td>Cord blood lymphocytes</td>
<td>LINE-1</td>
<td>r = 0.364 (p = 0.08)</td>
<td>Birth weight</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>
<td></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>
<td></td>
</tr>
<tr>
<td>Steegers-Theunissen et al. 2009³⁰²</td>
<td>Folic acid supplement, 400 µg/day</td>
<td>Periconceptional</td>
<td>Whole blood at 17 months old</td>
<td>IGF2</td>
<td>4.5% higher, (p=0.014)</td>
<td>Inverse association between IGF2 and birth weight (1.7% methylation per SD birth weight;</td>
</tr>
<tr>
<td>Fryer et al. 2011³³¹</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 CpG methylation correlated with plasma Hcy (p=0.04), and LINE-1 methylation (p=0.03)</td>
<td>CpG methylation correlated with birth weight percentile (P=0.02)</td>
</tr>
<tr>
<td>Ba et al. 2011³⁰⁴</td>
<td>Cord serum folate and B₁₂, 7.29 ng/ml Maternal serum folate and B₁₂, 2.29 ng/ml</td>
<td>N/A (Observational design)</td>
<td>Cord blood MNCs</td>
<td>IGF2 promoter2 IGF2 promoter3</td>
<td>No association with folate; P3 methylation associated with maternal serum B₁₂ (mean change=-0.22, p=0.0014)</td>
<td>Not determined</td>
</tr>
<tr>
<td>Hoyo et al. 2011³⁰³</td>
<td>Folic acid users vs. non-users before or during pregnancy</td>
<td>N/A (Observational design)</td>
<td>Cord blood leukocytes</td>
<td>IGF2 DMR H19 DMR</td>
<td>No association 2.8% decrease (p = 0.03) before pregnancy 4.9% decrease (p = 0.04) during pregnancy</td>
<td>Chronic diseases related to loss of IGF2 imprinting* (high methylation, low birth weight)</td>
</tr>
<tr>
<td>Study</td>
<td>One-carbon nutrient source &amp; levels</td>
<td>Duration</td>
<td>Tissue</td>
<td>DNA methylation</td>
<td>Effect on DNA methylation</td>
<td>Outcomes on Offspring</td>
</tr>
<tr>
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</tr>
<tr>
<td>Boeke et al. 2012</td>
<td>Dietary intakes of folic acid, B&lt;sub&gt;12&lt;/sub&gt;, choline, betaine</td>
<td>Pregnancy</td>
<td>Cord blood leukocytes</td>
<td>LINE-1</td>
<td>No association</td>
<td>Not determined</td>
</tr>
<tr>
<td>McKay et al. 2012</td>
<td>Maternal RBC folate, 379 ng/ml, Maternal B&lt;sub&gt;12&lt;/sub&gt;, 283 pg/ml</td>
<td>N/A (Observational design)</td>
<td>Cord blood</td>
<td>LUMA</td>
<td>Maternal B&lt;sub&gt;12&lt;/sub&gt; inversely associated with cord genome-wide DNA methylation (P=0.04)</td>
<td>Not determined</td>
</tr>
<tr>
<td>Haggarty et al. 2013</td>
<td>Folic acid supplement users</td>
<td>Before and after 12 weeks gestation</td>
<td>Cord blood</td>
<td>LINE-1, IGF2, PEG3, SNRPN</td>
<td>Supplementation &gt;12 wks gestation associated with -0.3% (P=0.03) +0.7% (P=0.04) -0.5% (P=0.02) No association</td>
<td>Genes related to fetal growth, imprinting syndromes, obesity, metabolic syndrome, tumour development, and neurogenetic disorders</td>
</tr>
<tr>
<td>van Mil et al. 2014</td>
<td>Folic acid supplement use Plasma folate, 19.1±9.0 nmol/L Plasma Hcy, median (90% range) 7.0 (4.9-10.9) µmol/L</td>
<td>N/A (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>
<td>Genes for fetal growth and neurodevelopment candidate genes</td>
</tr>
<tr>
<td>Azzi et al. 2014</td>
<td>Supplementation with folic acid alone, or the combo of folic acid, B&lt;sub&gt;6&lt;/sub&gt; &amp; B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Before or during pregnancy</td>
<td>Cord blood leukocytes</td>
<td>PLAG1</td>
<td>No association with PLAG1 or C-peptide; positively associated with estimated birth weight at 32 wk gestation (r=0.15 P=0.01), weight at birth (r=0.09 P=0.15), and weight at 1 yrs (r=0.14 P=0.03).</td>
<td>Birth weight (higher folate higher birth weight)</td>
</tr>
<tr>
<td>Hoyo et al. 2014</td>
<td>Quartiles of RBC folate concentrations</td>
<td>N/A (Observational design)</td>
<td>Cord blood leukocytes</td>
<td>IGF2, H19, DLK1/MEG3, PEG1/MEST, PEG3, PEG10/SGCE, PLAG1, NNAT</td>
<td>Q2 &lt; Q1 (P=0.004) No association Q4 &gt; Q1 (P=0.001) No association Q2 &gt; Q1 (P=0.03) No association Q3 &lt; Q1 (P=0.01) No association</td>
<td>Birth weight (higher folate higher birth weight)</td>
</tr>
<tr>
<td>Dominguez-Salas et al. 2014</td>
<td>Plasma folate, B&lt;sub&gt;12&lt;/sub&gt;, B&lt;sub&gt;6&lt;/sub&gt;, ACTB&lt;sub&gt;12&lt;/sub&gt; (holotranscobalamin), choline, betaine, methionine, homocysteine, SAM, SAH, DMG</td>
<td>N/A (Observational design)</td>
<td>Maternal blood near conception (one month prior to pregnancy)</td>
<td>BOLA3, LOX654433, EXD3, ZFYVE28, RMM46, ZNF67</td>
<td>Mean methylation higher in 2- to 8-month infants conceived in the rainy (nutrient-rich) season</td>
<td>Not determined</td>
</tr>
<tr>
<td>Adaikalakoteswari et al. 2015</td>
<td>Supplementation of folic acid and B&lt;sub&gt;12&lt;/sub&gt; dietary intake</td>
<td>N/A (Observational design)</td>
<td>Cord blood</td>
<td>Promoter regions of SREBF1 and LDLR ID11, SQLE, SC4MOL and INSIF1, StarD4</td>
<td>Hypomethylated and upregulated in response to B&lt;sub&gt;12&lt;/sub&gt; insufficiency</td>
<td>Genes associated with cholesterol biosynthesis and transport regulators</td>
</tr>
</tbody>
</table>

Table modified from Plumptre et al. 2016 thesis". 

2.4 The PREFORM Study

A recent prospective study, the PREnatal FOlic acid exposuRe on DNA Methylation in the newborn infant (PREFORM) Study, investigated the effect of maternal intakes and blood levels of one-carbon nutrients including folate/folic acid and B₁₂ on genome-wide DNA methylation in cord blood mononuclear cells (MNCs). The PREFORM study is the parent study from which this present study was conducted. Briefly, the PREFORM study aimed at determining the effects of maternal intakes and blood levels of one-carbon nutrients on genome-wide DNA methylation (5mC) and hydroxymethylation (5hmC) markers in cord blood MNCs. Assessment of baseline maternal and infant characteristics and blood concentrations occurred during early pregnancy (12-16 wks of gestation) and preconception (previous 3 months). A 110-food item modified and validated Block FFQ (NutritionQuest, Berkeley, CA) and Demographic and Health Questionnaire (DHQ) were used to assess baseline demographics and consisted of sections of the Canadian Community Health Survey (CCHS) cycle 2.2. Infant characteristics such as 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. RBC folate was assessed using Elecsys Folate Assay (Roche Diagnostics, Mannheim, Germany) and B₁₂ by Access competitive-binding immunoenzymatic assay (Beckman Coulter, Brea, CA). See Appendix A for analysis of blood biomarkers in maternal and umbilical cord blood.

A summary of RBC folate and serum B₁₂ concentrations at recruitment and delivery and in cord blood are shown in Table 2.4a. As shown, maternal and cord blood concentrations of RBC folate are high, while serum B₁₂ concentrations are shown to be adequate.

<table>
<thead>
<tr>
<th>Nutrient 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>RBC folate (nmol/L)</td>
<td>2416 (2361, 2472)</td>
<td>2789 (2717, 2864)</td>
<td>2681 (2607, 2758)</td>
</tr>
<tr>
<td>Serum B₁₂ (pmol/L)</td>
<td>218 (208, 227)</td>
<td>168 (161, 175)</td>
<td>320 (299, 342)</td>
</tr>
</tbody>
</table>

Reprinted with permission from Plumptre et al. 2015.
The main findings of the PREFORM study have been published previously in Plumptre et al. 2015 and Visentin et al 2016\textsuperscript{143,204}. This study found that maternal serum B\textsubscript{12} concentrations in early pregnancy were positively correlated with 5mC content in cord blood MNCs ($r=0.25$, $p=0.002$), while RBC folate concentrations in early pregnancy were positively correlated with 5-hmC ($r=0.16$, $p=0.04$). Additionally, plasma betaine concentrations ($r=-0.3$, $p=0.01$) in early pregnancy were positively, whereas cord plasma UMFA concentrations ($r=-0.23$, $p=0.004$) at early pregnancy were negatively, correlated with 5-hmC content. In a sub-analysis, mothers with early pregnancy RBC folate concentrations in the highest folate quartile (>2860 nmol/L) and serum B\textsubscript{12} (<167 pmol/L) concentrations in the lowest quartile had significantly lower 5mC content in cord blood MNC and higher birth weight in comparison with those with lower RBC folate (quartiles 1-3) and higher serum B\textsubscript{12} (quartiles 2-4) concentrations (Table 2.4b).

Table 2.4b. Genome-wide DNA methylation and hydroxymethylation and infant birth weight

<table>
<thead>
<tr>
<th></th>
<th>High Folate/Low B\textsubscript{12} (n=13)</th>
<th>Moderate Folate/Marginal to Adequate B\textsubscript{12} (n=266)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant 5mC</td>
<td>5.16 ± 0.28 %</td>
<td>5.50 ± 0.41 %</td>
<td>0.0069</td>
</tr>
<tr>
<td>Infant 5hmC</td>
<td>0.025 ± 0.015%</td>
<td>0.032 ± 0.016%</td>
<td>0.208</td>
</tr>
<tr>
<td>Infant Birth Weight</td>
<td>3697 ± 455 g</td>
<td>3393 ± 451 g</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*p-value represents statistical significance of differences in cord MNCs 5mC, 5hmC content and infant birth weight between groups of mothers using a t-test. High RBC folate >2860 nmol/L (quartile 4), low B\textsubscript{12} <167 pmol/L (quartile 1). Quartiles 1-3 and 2-4 belong to the moderate folate/marginal to adequate B\textsubscript{12} group.

In summary, the literature of my thesis highlights the relationship between nutrient exposure of folate and B\textsubscript{12} and infant health and disease through DNA methylation. Few studies have investigated this tripartite relationship, specifically in combination with folate and B\textsubscript{12}, in humans. Also, few studies have investigated this in a gene-specific manner in cord blood MNCs. As the PREFORM study examined the effects of genome-wide DNA methylation and hydroxymethylation on one-carbon nutrients, this study takes a step further and address the gaps in the literature by investigating folate and B\textsubscript{12} on gene-specific DNA methylation and its biological and clinical functional relevance to infant health and disease.
3- Rationale, Research Questions, Hypothesis and Objectives

3.1 Rationale

Folate and folic acid intakes and blood concentrations are higher presently in Canada than they have been in the past 20 years\textsuperscript{65,133}. This is mainly attributed to both the 1998 mandatory folic acid fortification and widespread use of folic acid supplements\textsuperscript{193}. In addition, WCBA are advised to take folic acid supplement before and during pregnancy. The Canadian and US nationwide data indicate that the majority of women take prenatal supplements mostly containing 1000 µg folic acid\textsuperscript{137}. Similarly, B\textsubscript{12} intakes are shown to be adequate in pregnant women through intake of prenatal supplements containing B\textsubscript{12}. Nevertheless, studies including our PREFORM study\textsuperscript{135,204}, have found that despite adequate intakes, marginal to inadequate B\textsubscript{12} concentrations were seen in pregnant women\textsuperscript{203,207}. Exposure to high folate and low B\textsubscript{12} has been associated with adverse maternal and offspring health outcomes. Particularly, low B\textsubscript{12} amidst a high folate environment have been related to an increased risk for small-for-gestational-age babies\textsuperscript{12} and childhood adiposity and insulin resistance at age six\textsuperscript{11}. Mothers with the highest folate and lower B\textsubscript{12} blood concentration group reported to have children who were the most insulin resistant\textsuperscript{11}. Despite these findings, current evidence is insufficient to establish strong associations between maternal folate and B\textsubscript{12} status and health outcomes. Folate and B\textsubscript{12} are integral factors in the one-carbon metabolic pathway that have the potential to affect DNA methylation through modulation of methionine synthase activity and the concentration of S-adenosyl methionine, the universal methyl group donor for DNA methylation and over 100 other biological methylation reactions\textsuperscript{45}. DNA methylation is an epigenetic process that affects gene expression and stability, and is therefore important in human health and disease susceptibility\textsuperscript{252}. The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that intrauterine environmental exposures can influence chronic disease susceptibility in the offspring later in life through hormonal, metabolic or epigenetic changes\textsuperscript{268}. DNA methylation is programmed in embryogenesis in order to establish a unique DNA methylation pattern in the fetal genome\textsuperscript{6}. Animal studies have shown that
maternal diet, including one-carbon nutrients, can alter DNA methylation in the offspring and affect permanent phenotypic changes and disease susceptibility related to birth weight, adiposity and metabolic disorder. Human studies have also shown that maternal diet, including one-carbon nutrients, can alter DNA methylation patterns in the offspring and affect infant health in terms of birth weight, metabolism, and developmental processes. However, the role of genome-wide and gene-specific DNA methylation in linking intrauterine environmental dietary exposure and infant health and disease has not been clearly elucidated.

Given the aforementioned considerations and access to DNA from the PREFORM study, the overarching research question is: Can maternal B12, in combination with a high folate environment have an epigenetic effect in the offspring, and influence infant health and disease outcomes?
3.2 Hypothesis

I hypothesize that:

1) Low maternal B₁₂ will decrease gene-specific DNA methylation patterns in cord blood MNCs in a high folate environment.

2) CpG methylation alterations associated with maternal B₁₂ and folate status will be observed primarily in genes related to intrauterine growth and development.

3) The observed CpG methylation alterations involved in intrauterine growth and development will affect the expression of these genes.

3.3 Objectives

1) To determine gene-specific DNA methylation alterations in newborn infants born to mothers with low B₁₂ status in comparison to those born to mothers with higher B₁₂ status in a high folate environment.

2) To determine functional, biological, and clinical ramifications of altered gene-specific DNA methylation patterns associated with the maternal low B₁₂ and high folate status.
4- Effects of Maternal Vitamin B\textsubscript{12} Status in Combination with High Folate Status on Gene-Specific DNA Methylation in Cord Blood Mononuclear Cells

4.1 Subjects and Methods

4.1.1 Study Design and Sample Selection for the Present Study

Using the PREFORM cohort, mothers were separated to cases and controls based on serum B\textsubscript{12} and RBC folate blood concentrations and matched based on a specific criteria shown in Table 4.1.1. The matched cord blood DNA samples were analyzed for gene-specific DNA methylation using Illumina Infinium MethylationEPIC array. CpG sites and their associated genes were identified with bioinformatics analysis for methylation levels in cases and controls. Ingenuity Pathway Analysis was used for functional analysis to identify biological, disease and physiological functions that are most significant to genes that are differentially methylated. mRNA expression for candidate genes that are shown to be regulated by DNA methylation was confirmed by Droplet Digital PCR (ddPCR) (Figure 4.1.1a). Cord blood MNCs RNA extraction, quantity and quality were determined using NanoDrop-1000 (Thermo Scientific) Spectrophotometer and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA).

**Figure 4.1.1a. Study Design.** Baseline characteristics, dietary and blood sample data at early pregnancy was obtained from the PREFORM study. Data was used for sample selection, DNA methylation, functional and gene expression analyses. Adapted with permission from Plumptre et al. 2015\textsuperscript{143}.
A total of 44 women were selected from the original PREFORM cohort using the propensity score. From the 364 mothers, 15% (n=55) were lost to follow up mainly due to their transfer to another centre to give 309 mother-child pairs (Figure 4.1.1b). They were matched based on RBC folate and serum B₁₂ concentration cutoffs as well as a specific matching criterion. Cases were selected based on ‘inadequate’ serum B₁₂ blood concentrations that are <148 pmol/L and controls based on ‘adequate’ B₁₂ >148 pmol/L. This cutoff is used as a diagnostic cutpoint for B₁₂ deficiency based on the 1999-2004 NHANES and is currently used in the CHMS. RBC folate concentrations were >1800 nmol/L for both cases and controls. This cutoff is based on 1820 nmol/L post-fortification RBC folate concentration of the 90th percentile of the 2005—2010 NHANES. Using this cutoff allows for more consistent measurement of a high folate status in WCBA, and subsequently, better evaluation of their health outcomes and their potential offspring. Furthermore, in this present study, 1800 nmol/L cutoff was used over a lower cutoff because of the generally very high RBC folate concentration of mothers in the PREFORM study. A higher cutoff was also not chosen in order to be more generalizable to cutoffs found in the literature investigating maternal RBC folate status and offspring health and disease outcomes. Propensity score matching using SAS 9.4 (SAS Institute Inc., Cary, NC) was used to control for potential confounding variables for DNA methylation between the cases and controls and determine the final number of matches (Table 4.1.1). Missing DNA and RNA samples were removed from the matches, and together with a final propensity score model matched by case to control ratio of 1:1, gave a total of 44 matched samples.
Seven variables were chosen a priori and controlled for by the propensity score as they have been shown in the literature to affect DNA methylation. These variables are maternal plasma choline and B<sub>6</sub> concentrations at early pregnancy, maternal age, maternal BMI at early pregnancy, mode of delivery and infant sex (Table 4.1.2). The Infant gestational age was removed from the matching criteria as all preterm births <37 weeks were removed from the cohort. The remaining two variables of maternal ethnicity and smoking during pregnancy were not controlled for as it did not satisfy the Standardized Differences Calculation for continuous variables test and it restricted the propensity score matches.

Figure 4.1.1b. Final sample selection and flowchart of the PREFORM participants. Cases and controls were matched based on specific criteria from the original PREFORM cohort of 309 mother-child pairs. Adapted with permission from Plumptre et al. 2016.
Table 4.1.1. Matching criteria for cases and controls

<table>
<thead>
<tr>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age</td>
</tr>
<tr>
<td>Maternal BMI</td>
</tr>
<tr>
<td>Maternal plasma $B_6$</td>
</tr>
<tr>
<td>Maternal plasma Choline</td>
</tr>
<tr>
<td>Mode of Delivery</td>
</tr>
<tr>
<td>Infant Sex</td>
</tr>
<tr>
<td>Infant Gestational Age</td>
</tr>
<tr>
<td>Maternal Ethnicity</td>
</tr>
<tr>
<td>Pregnancy Smoking</td>
</tr>
</tbody>
</table>

The way the propensity score matches cases to the controls is through an algorithm that uses a ‘caliper’ value that is +/- 0.2*standard deviation. The propensity score also provides a c-statistic as a measure of how well the criteria, expressed in a regression equation, predicts case or control. Values range from 0.5 to 1.0, where a value close to 1 indicates that the cases and controls are very different from one another and thus any differences in their outcomes may be due to differences in other variables instead of folate and $B_12$. A c-Statistic of 0.7 was produced by the final propensity score model shown below. This c-statistic indicates that cases and controls are quite different; nevertheless, there is a fair amount of overlap in the propensity scores between them. The final propensity score model used to generate the matched case with controls and the total number of matches is shown in Appendix B.

In order to produce this final propensity score model with a c-statistic of 0.7, all nine variables were assessed for redundancy to ensure that none of them was a restatement of the other. This was assessed through understanding of each variable biologically and more formally with statistical tests. Chi-Squared tests for dichotomous and T-tests for normally distributed continuous variables found significance with pregnancy smoking (p = 0.0675), BMI (p=0.0507), and $B_6$ (p=0.0002). Other variables were non-significant with ethnicity (p=0.2233), infant sex (p=0.4187), mode of delivery (p=0.2432), maternal age (p=0.7042), and choline (p=0.9136). Hence, these three variables were the focus of the propensity score regression model equation to be controlled. Additionally, a Standardized Difference calculation was performed in order to verify whether the propensity score matched accurately.
A three-interaction term was created in the regression model equation in order to control for pregnancy smoking; however, the propensity score was unable to effectively control for this variable as the Standardized Difference was > 0.1. Similarly, maternal ethnicity and BMI did not match accurately. BMI was thus included in the ‘mvars’ term of the propensity score with a ‘dmaxk’ of 3. ‘Mvars’ creates BMI as a covariate to the propensity score where the ‘dmaxk’ uses an exact matching method with a range of 3 to pair cases with control. As BMI is depicted with ranges for each lean, overweight, and obese categories, matching a ‘dmaxk’ by 3 is clinically acceptable. The two-way interaction terms were not included in the regression model as the purpose was solely for matching and not for model creation.

4.1.2 Genome-Wide and Gene-Specific DNA Methylation and Functional Analyses

4.1.2.1 Illumina Infinium MethylationEPIC Array

The Illumina Infinium MethylationEPIC array was used for gene-specific methylation analysis, interrogating 850,000 CpG methylation sites quantitatively across the genome at single-nucleotide resolution for >99% of RefSeq genes\textsuperscript{347}. DNA of each sample were processed at the SickKids TCAG Core Facility (Toronto, ON). Briefly, 1 µg of genomic DNA was bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research) according to manufacturer’s instructions. Unmethylated cytosines are converted to uracil in the presence of bisulfite, while methylated cytosines are unaffected by deamination of bisulfite and remain as cytosine. The bisulfite conversion included a thermoycling program with a short denaturation step (16 cycles of 95°C for 30 seconds followed by 50°C for 1 hour). The amount of bisulfite-converted DNA and completeness of bisulfite conversion were assessed using a panel of MethylLight-based quality control (QC) reactions as previously described\textsuperscript{349}. All samples passed QC tests and were used for the Infinium HD Methylation Assay pipeline for whole-genome amplification (WGA) and endpoint enzymatical fragmentation. The bisulfite-converted WGA-DNA samples were purified and applied to the BeadChips and were incubated in order to hybridize. During hybridization, the WGS-DNA molecules annealed to locus-specific DNA oligomers linked to individual bead types. The two bead types
corresponded to a methylated (C) or unmethylated (T) state of each CpG locus. This allele-specific primer annealing was followed by single-base extension of detectable labels of DNP- and Biotin-labeled ddNTPs. Both bead types for the same CpG locus incorporated the same type of labeled nucleotide, determined by the base preceding the interrogated “C” in the CpG locus, and therefore would be detected in the same color channel (Figure 4.1.2.1). After extension, the BeadChip was scanned using a HiScan System and the intensities of the unmethylated and methylated bead types measured from the light emitted from the fluorophores.

Figure 4.1.2.1. The HD Infinium assay for methylation. Methylation status at each CpG loci is determined by M or U bead-bound probes that detect presence of C or T of the bisulfite-converted DNA. Methylated C is protected from conversion (left), while unmethylated C is converted to T (right). By hybridization, methylated and unmethylated bead-bound probes are used to identify CpG loci methylation. This is followed by single-base extension with a labelled nucleotide. (https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/humanmethylationepic-data-sheet-1070-2015-008.pdf)

4.1.2.2 Bioinformatics

A measure of the level of DNA methylation at each CpG site was scored as beta (β)-values using IlluminaGenomeStudio 2011.1 software (Illumina Inc., San Diego, CA). DNA methylation β-values represent the ratio of the intensity of the methylated bead type to the combined (methylated and unmethylated) locus intensity ranging from 0 to 1. Values close to 0 indicate low levels of methylation, while values close to 1 indicate high levels of DNA methylation. Raw scanned data were normalized and
filtered based on detection p-values, non-CpG sites, cross-reactive probes, and sex chromosomes giving 804, 449 CpG sites. The detection p-values for each β-value measure the difference of signal intensities at the interrogated CpG site and is compared to the standard deviation of 600 negative control probes to establish detection limits for methylation probes. All data points with a detection p-value >0.05 were considered not statistically significantly different from background measurements, and therefore filtered out to ensure inclusion of only high-confidence probes. CpG sites that are differentially methylated were determined using a Mann-Whitney non-parametric test due to the bimodal distribution of having either hyper- and hypomethylated patterns. Total CpG site data were filtered to 921 CpG sites (521 CpG-site-associated genes) by methylation difference score (DiffScore) ≥13 and ≤-13 and delta β-values ≥0.1 and ≤-0.1 based on intergroup differences in methylation levels between cases and controls. Average β-values for each case and control group is a measure of the ratio of signal from the methylated probe to the sum of both methylated and unmethylated signals. A Student’s T-test was used to obtain 98 CpG sites (69 CpG-site-associated genes) that differed most between cases and controls, where statistical significance was assessed with p-values and corrected for multiple comparison testing using a Benjamini-Hochberg correction giving q-values. A q-value is an ‘adjusted p-value’ indicating the level of false positives for a given p-value and is thus used to control for false positives. A q-value greater than 0.05 implies that more than 5% of significant tests are false positives. Fold change was used to determine methylation status of each CpG site. Fold change represents the average difference to the power of two between cases and controls that are part of the T-test. Statistical analysis was carried out using the SAS 9.4 software. The Illumina Infinium MethylationEPIC DNA methylation β-values were represented graphically using heat maps and boxplots generated by the Qlucore Omics Explorer (Qlucore, New York, NY) and GenomeStudio 2011.1 softwares.
4.1.2.3 Gene Selection for Gene Expression Analysis

Based on bioinformatic and functional analyses, 7 hypomethylated and 7 hypermethylated genes in the cases were selected for gene expression analysis. The genes were selected based on the most significant DiffScore, greatest magnitude delta β-values, and most relevant biological functions to growth and development.

4.1.3 Functional Analysis

4.1.3.1 Ingenuity Pathway Analysis

The functional analysis was performed using Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, Redwood City, CA; http://www.ingenuity.com) to identify diseases, networks, biological functions and genes that were most differentially methylated between cases and controls. The Ingenuity Knowledge Base contains findings and annotations from the literature as well as other sources including EntrezGene, RefSeq, OMIM, GWAS database, Gene Ontology, Tissue Expression Body Atlas, NCI-60 Cell Line Expression Atlas, KEGG Metabolic pathway information. The right-tailed Fisher’s exact test was used to calculate a p-value determining the likelihood that each biological function, network and/or disease assigned to the focus data set is due to chance alone. For network analysis, IPA computes a score for each network according to the fit of that network to the user-defined data set of focus genes based on a p-value that is displayed as the negative log of that value. The score indicates the likelihood of the focus genes in a network being found together due to random chance, where a score of 2 indicates that there is a 1 in 100 chance that the focus genes in a network are due to chance. Hence, scores of 2 or higher have at least a 99% confidence of not being due to random chance alone.
4.1.4 Gene Expression Analysis

4.1.4.1 RNA Extraction

RNAs from cord blood MNCs were extracted using RNeasy® Mini Kit (Qiagen, Cat#: 74104) according to the manufacturer’s protocol. The protocol involved loosening MNCs by passing lysate through a 20-gauge needle in a phenol/guanidine-based lysis buffer and subsequently with ethanol. RNA was recovered using a RNA-binding-specific silica membrane within a spin-column. On-column DNA digestion using the RNase-free DNA set (Qiagen) was also performed to ascertain prevention of DNA contamination within the samples. RNA concentration was measured using a NanoDrop-1000 Spectrophotometer through measurement of pertinent absorbance values and calculation of absorbance ratios. All samples had a ratio of A260:A280 nm between 2.0 and 2.09, indicating pure RNA. The purified RNA was stored at -20°C for further analysis.

RNA concentration and integrity was assessed using the Agilent RNA 6000 Nano Kit and the 2100 Bioanalyzer (Agilent Technologies) instrument, a microfluidics-based platform, was used for quantification and quality of RNA. The RNA Integrity Number (RIN) score was generated on the Agilent software. For gene expression analysis, 26 cord lymphocytes samples were available and only 8 samples had more usable RINs. The RNA quality for the 4 cases matched to its 4 controls had variable RINs, ranging from 3.5 to 9.7, and was attributed to the collection and storage variability upon collection of cord blood MNCs at birth.

4.1.4.2 Droplet Digital Polymerase Chain Reaction

cDNA was synthesized using the QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer’s protocol. Droplet Digital™ Polymerase Chain Reaction (ddPCR) was performed using the QX200™ Droplet Digital™ PCR System (Bio-Rad) according to the manufacturer’s instructions. Briefly, ddPCR is a technique that partitions a sample into 20,000 smaller reaction vessels or ‘droplets’ and
measures fluorescence of the amplified target gene in each droplet. Fluorescence measurement thus gives a positive and negative population of binary readouts, where ones (positive) indicates gene presence and zeroes (negative) indicates gene absence. Since the presence of a gene in a given droplet is a random event, the data generated fits a Poisson distribution. This removes the need for standard curves and allows for a robust estimation of data dispersion from technical replicates in absolute quantities\textsuperscript{352}. Compared to RT-qPCR, ddPCR allows for increased sensitivity and accuracy in detecting gene expression when the gene concentration is low and when the gene expression is previously known to be low in the target cell- or tissue-species. Furthermore, absolute quantification removes the necessity for a housekeeping gene.

ddPCR reactions were performed in a 10 µl volume with EvaGreen with ddPCR supermix (Bio-Rad), 2 µl of cDNA template (1 ng/µl), and 2 µl of candidate gene primers diluted 4X to 2.5 µM for \textit{FRMD4B}, \textit{ARGHEF12}, and 10X to 1.25 µM for \textit{HLAE}, \textit{PRKCH}, \textit{SLC25A24} and \textit{CYFIP1}. Primers and their forward and reverse sequences are shown in (\textbf{Table 4.1.4.2}). cDNA and primer concentrations and volumes were optimized through repeated adjustments until ‘rain’ or noise in the ddPCR plots were minimized and all samples had approximately 20,000 droplets. Negative controls that do not contain sample cDNA should have small fluorescence ranges for the negative population and positive population with low or no droplets. 20µl of the ddPCR reaction was loaded into the droplet generator to partition the sample into droplets. The cycling conditions included: 2 mins at 50°C and 10 mins at 95°C which was followed by 40 cycles involving 15 second denaturation at 95°C and 1 min primer annealing and fragment elongation at 62°C. Three genes (\textit{DOCK10}, \textit{SCD} and \textit{APBB2}) were run in slightly different conditions with 61°C annealing for 1 minute for optimized runs. QuantaSoft software (Bio-Rad, Hercules, CA) was used for analysis of fluorescence based on positive and negative readings in each sample. The fraction of positive droplets determines concentration of gene in copies/µl\textsuperscript{353}. The mean (± stdev) concentration of gene copies/µl was obtained from the 8 cases and 8 control samples, and their difference was calculated for each gene.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HLA-E</strong></td>
<td>Forward: 5’-AGC TGT CTC AGG CT  T TAC AAG-3’&lt;br&gt;Reverse: 5’-CTG TGA TAT GGA GGA AGA AGA GC-3’</td>
</tr>
<tr>
<td><strong>LIF</strong></td>
<td>Forward: 5’-TCC AGT GCA GAA CCA ACA G-3’&lt;br&gt;Reverse: 5’-GAG CAT GAA CCT CTG AAA ACT G-3’</td>
</tr>
<tr>
<td><strong>FRMD4B</strong></td>
<td>Forward: 5’-GTT TCC ATC TGT ACC ATC CAG T-3’&lt;br&gt;Reverse: 5’-TCC AGC ACA TTC TTT TTA CAC C-3’</td>
</tr>
<tr>
<td><strong>MYT1L</strong></td>
<td>Forward: 5’-GCT CTG TGC TAT CCT GAT ACC -3’&lt;br&gt;Reverse: 5’-GCT GTG ATG GTT CTG GAC AT-3’</td>
</tr>
<tr>
<td><strong>CYFIP1</strong></td>
<td>Forward: 5’-CAT GCA GAT ACT GAG GCT GT-3’&lt;br&gt;Reverse: 5’-CTA TGA CTT CCT GCC CAA CTA C-3’</td>
</tr>
<tr>
<td><strong>GRIN1A</strong></td>
<td>Forward: 5’-GCA GAA ACA ATG AGC AGC ATC-3’&lt;br&gt;Reverse: 5’-CAA GAA GTA ATG GCA CGG TCT-3’</td>
</tr>
<tr>
<td><strong>DOCK10</strong></td>
<td>Forward: 5’-GCT GTG CTG CAT CTT CCT GAT CTA CTG-3’&lt;br&gt;Reverse: 5’-GCT GTG CTG CAT CTT CCT GAT CTA CTG-3’</td>
</tr>
<tr>
<td><strong>PPAP2B</strong></td>
<td>Forward: 5’-GAA CCT GTC CAT CTT GAT GTA ATG GCA AAA CC-3’&lt;br&gt;Reverse: 5’-TCG ACC TCT TCT GCC TCT T T-3’</td>
</tr>
<tr>
<td><strong>SCD</strong></td>
<td>Forward: 5’-GGA ATT ATG AGT ATT GAT AGT TG-3’&lt;br&gt;Reverse: 5’-GCA ATT ATG AGT ATT GAT AGT TG-3’</td>
</tr>
<tr>
<td><strong>ARHGEF12</strong></td>
<td>Forward: 5’-ACC AGA GTT CCA TTC ACC TTG -3’&lt;br&gt;Reverse: 5’-ACA ATC CAG TCT TCG TAC AGT-3’</td>
</tr>
<tr>
<td><strong>SLC24A24</strong></td>
<td>Forward: 5’-GAG CAT TGC TTG TTC GAG AGA CA -3’&lt;br&gt;Reverse: 5’-GCT TAA CTA TTC CAG ATG AAT TCA CG-3’</td>
</tr>
<tr>
<td><strong>PRKCH</strong></td>
<td>Forward: 5’-GTT CTT CAT CAA AAC GAC GAG -3’&lt;br&gt;Reverse: 5’-CAG TTG TTC TGC TGC TTT CAG -3’</td>
</tr>
<tr>
<td><strong>APBB2</strong></td>
<td>Forward: 5’-CTG ATG CCA CTG TGA CTG TC-3’&lt;br&gt;Reverse: 5’-CAT GAT GAA GGC AAA TGT GTG G-3’</td>
</tr>
</tbody>
</table>
4.1.5 Statistic Analysis

For sample selection of maternal dietary and supplemental intakes, and blood concentrations, a paired T-test (for continuous variables) and a McNemar’s test (for categorical characteristics) was used for comparison between case and control means. A Wilcoxon Signed-Rank Sum test was used for skewed distributions. For genome-wide DNA methylation CpG and non-CpG island analyses, statistical significance was determined using a paired T-test for comparison of average β-values for case and control. The results were considered statistically significant if the p-values were < 0.05. A Wilcoxon Signed-Rank Sum test was also used for total and non-CpG island methylation due to its skewed distribution. Analyses and graphical representations were done using GenomeStudio 2011.1 and SAS 9.4. For gene-specific methylation, detailed information on data filtering, normalization and statistical analysis is presented in Section 4.1.2.3. Quilcore Omics Explorer was used to calculate the largest difference between the cases and controls, and a paired T-test was used to compare difference between the groups. For gene expression analysis for ddPCR, results were analyzed using mean copy number difference using a paired T-test.
4.2 Results

4.2.1 Subject Characteristics

4.2.1.1 Matched Cases and Controls

From the original PREFORM cohort of 309 women, 22 cases were matched to 22 controls based on RBC folate and serum B₁₂ concentration cutoffs as well as the specific matching criteria. Cases were selected based on ‘inadequate’ B₁₂ blood concentrations that are <148 pmol/L and controls based on ‘adequate’ B₁₂ >148 pmol/L. RBC folate concentrations were >1800 nmol/L for both cases and controls. Mean dietary and supplemental intakes and blood concentrations of folate and B₁₂ in early pregnancy for cases and controls are shown in Table 4.2.1.2 b and c.

4.2.1.2 Maternal and Infant Characteristics

Baseline maternal characteristics for cases and controls are listed in Table 4.2.1.2a. Maternal age, BMI, and plasma B₆ and choline concentrations were matched based on propensity score. On average, mothers were 32 years of age and had a BMI of 27 at recruitment. The ethnicities of mothers were Caucasian (59%), Asians (16%) included Chinese, Filipino, Japanese, Korean and Southeast Asian, Latin Americans (9%), and other ethnicities (16%) consisted of Black and Aboriginal. Twenty-three % of women reported completing a college/vocational diploma and 59% completed a university degree or more and are thus generally well educated. Few reported household income below the poverty line (9%). Even fewer were single mothers (7%). Seventy percent of mothers experienced their first pregnancy (gravidity = 1) and 36% were nulliparous (parity = 0). Mothers who smoked during pregnancy were minimal (7%). No significant differences were found between cases and controls using a paired T-test and a McNemar’s test.
Table 4.2.1a. Baseline maternal characteristics

<table>
<thead>
<tr>
<th>Descriptive characteristics</th>
<th>Cases (n=22)</th>
<th>Controls (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) [mean (95% CI)]*</td>
<td>31 (29, 34)</td>
<td>33 (31, 34)</td>
</tr>
<tr>
<td>Gestational Age at recruitment (wks)</td>
<td>13.3 (12.5, 14.0)</td>
<td>13.5 (12.6, 14.4)</td>
</tr>
<tr>
<td>BMI at recruitment (kg/m^2) [mean (95% CI)]*</td>
<td>26.7 (24.3, 29.2)</td>
<td>26.7 (24.1, 29.2)</td>
</tr>
<tr>
<td>Ethnicity (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Asian</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Latin American</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Born in Canada (n)</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Education Level (highest degree/diploma attained) (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High School Diploma</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>College/Vocational Diploma</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>University degree or higher</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Household income below poverty line (n)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Single parent family</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Gravidity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Parity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>&gt;1</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>Smoker</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Marked (*) characteristics belong to the matching criteria used in the propensity score analysis to matched cases with controls. p > 0.05 based on a paired T-test and a McNemar’s test.

Maternal dietary intakes and blood concentrations in early pregnancy are listed in Table 4.2.1b and c.

Relative dietary intakes (naturally occurring and folic acid as a fortificant) without supplementation of one-carbon nutrients, except for B₁₂, were shown to be higher in the cases compared to the controls. Statistical significance was found using a paired T-test for folate with a mean difference of 218 µg DFE/d (p=0.004), B₆ with 0.7 mg/d (p=0.01), and choline with 113 mg/d (p=0.006). B₁₂ was not significantly different between cases and controls (p > 0.05). Focusing mainly on folate and B₁₂ relative intakes, prevalence of inadequacy from diet alone during pregnancy was 18 of 44 (41%) and 3 of 44 (7%), respectively.

However, adequate folate and B₁₂ intakes were observed in all mothers owing to the use of prenatal supplement during pregnancy. Supplemental intakes (mean (95%CI)) of folic acid were 815 µg/day (578, 1052) for cases and 1423 µg/day (788, 2058) for controls. For B₁₂, they were 11.9 (8.9, 32.6) µg/day for
cases and 5.0 µg/day (2.9, 7.1) for controls. For B₆, supplemental intake was also adequate with 1.8 mg/d (0.9, 2.8) for cases and 3.9 mg/d (2.1, 5.8) for controls. There was no recorded information on choline supplemental intake as it was not included in the supplements. Based on paired T-tests for folic acid, B₁₂ and B₆, there were no significant differences observed between case and control supplemental intakes (p>0.05).

Table 4.2.1.2b. Maternal dietary intakes in early pregnancy

<table>
<thead>
<tr>
<th>Dietary Intake</th>
<th>Cases [Mean (95% CI)]</th>
<th>Controls [Mean (95% CI)]</th>
<th>EAR for Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (µg DFE/d)*</td>
<td>674 (564, 785)</td>
<td>456 (399, 512)</td>
<td>520</td>
</tr>
<tr>
<td>B₁₂ (µg/d)</td>
<td>5.6 (4.3, 7.0)</td>
<td>4.4 (3.6, 5.1)</td>
<td>2.2</td>
</tr>
<tr>
<td>B₆ (mg/d)*</td>
<td>2.2 (1.8, 2.7)</td>
<td>1.5 (1.3, 1.7)</td>
<td>1.6</td>
</tr>
<tr>
<td>Choline (mg/d)*</td>
<td>353 (286, 420)</td>
<td>250 (216, 264)</td>
<td>450 (AI)</td>
</tr>
</tbody>
</table>

DFE, dietary folate equivalent; EAR, estimated average requirement; AI, adequate intake.
* p<0.05 based on a paired T-test

In early pregnancy, overall use of prenatal B-vitamin supplements containing folic acid, B₁₂, B₆, other vitamins and minerals for cases and controls were 71% and 91%. Use of B vitamin-containing supplement of folic acid, B₁₂, B₆ containing supplement designed for non-pregnant adults at least once each week for cases and controls was 81% and 100%, multivitamins 5% and 0%, and folic acid supplementation alone 15% and 9%, respectively. The median supplemental intakes reported during early pregnancy was 1000 µg/d of folic acid and 2.6 mg/d of B₁₂ for cases and controls. Women consuming a combination of ≥2 of the B vitamin–containing supplements were >77% for both cases and controls.

Adequacy of nutrient status, considering dietary and supplemental intakes, are assessed using blood concentrations. Mean (95%CI) RBC folate and serum B₁₂ concentrations in early pregnancy were 2682 nmol/L (2506, 2858) and 120 pmol/L (112, 128) for cases and 2512 nmol/L (2324, 2700) and 253 pmol/L (220, 286) for controls, respectively. Folate, B₆ and choline are above while only B₁₂ control are below the inadequacy cutoffs. Serum B₁₂ concentration was significantly different between cases and controls with a mean difference of -133 pmol/L (p<0.0001).
Table 4.2.1.2c. Maternal blood concentrations in early pregnancy

<table>
<thead>
<tr>
<th>Blood Concentration</th>
<th>Cases [Mean (95% CI)]</th>
<th>Controls [Mean (95% CI)]</th>
<th>Inadequacy Cutoff</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC folate (nmol/L)</td>
<td>2682 (2506, 2858)</td>
<td>2512 (2324, 2700)</td>
<td>&lt;305</td>
</tr>
<tr>
<td>Serum B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)*</td>
<td>120 (112, 128)</td>
<td>253 (220, 286)</td>
<td>&lt;148</td>
</tr>
<tr>
<td>Plasma B&lt;sub&gt;6&lt;/sub&gt; (nmol/L)*</td>
<td>81 (67, 95)</td>
<td>81 (58, 103)</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Plasma choline (nmol/mL) *</td>
<td>7.2 (6.7, 7.9)</td>
<td>7.1 (6.4, 7.8)</td>
<td>n/d</td>
</tr>
</tbody>
</table>

Marked (*) characteristics belong to the matching criteria used in the propensity score analysis to matched cases with controls. n/d (not determined); there is currently no set cutoff for plasma choline.<sup>304</sup> p<0.0001 based on a paired T-test.

Newborn characteristics are listed in Table 4.2.1.2d. Overall, all newborns are born full term (>37 weeks gestation) with normal Apgar score. Infants in the case group are shown to have lower Apgar five (p=0.03) scores based on the Wilcoxon Signed-Rank Sum test, and lower Apgar one (p=0.05) based on a paired T-test. There were no significant differences in birth weight, head circumference and birth length between cases and controls. Birth weight percentile was found to be borderline significant based on the Wilcoxon Signed-Rank Sum test with a p-value of 0.055. None were diagnosed with gestational diabetes and pregnancy induced hypertension, but one woman had an NTD-affected pregnancy.

Table 4.2.1.2d. Newborn characteristics

<table>
<thead>
<tr>
<th>Descriptive characteristics</th>
<th>Case (n=22)</th>
<th>Controls (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at birth (wks)*</td>
<td>39.8 (39.3, 40.3)</td>
<td>39.9 (39.5, 40.3)</td>
</tr>
<tr>
<td>Caesarian delivery, n*</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Male, n*</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3634 (3429, 3840)</td>
<td>3465 (3304, 3627)</td>
</tr>
<tr>
<td>Birth weight percentile (%)*</td>
<td>80 (70, 89)</td>
<td>69 (58, 80)</td>
</tr>
<tr>
<td>Birth length (cm)</td>
<td>52.9 (51.7, 54.2)</td>
<td>52.4 (51.3, 53.6)</td>
</tr>
<tr>
<td>Head circumference (cm)</td>
<td>34.7 (33.9, 35.4)</td>
<td>34.2 (33.7, 34.8)</td>
</tr>
<tr>
<td>1 min. Apgar score*</td>
<td>7.9 (6.9, 8.9)</td>
<td>8.9 (8.8, 9.0)</td>
</tr>
<tr>
<td>5 min Apgar score*</td>
<td>8.6 (7.9, 9.3)</td>
<td>9.1 (8.9, 9.3)</td>
</tr>
</tbody>
</table>

Marked (*) characteristics belong to the matching criteria used in the propensity score analysis to matched cases with controls. Values expressed at mean (95% CI).

*p ≤ 0.05 based on a Wilcoxon Signed-Rank Sum test for 5 min Apgar score

*p ≤ 0.05 paired T-test for 1 min Apgar score and birth weight percentile.
4.2.2 Genome-wide DNA Methylation

Since the Illumina Infinium MethylationEPIC array contains additional probes that are in non-CpG islands, which are more hypermethylated\(^\text{10}\) (see Appendix C), a separate genome-wide methylation was performed for CpG islands and non-CpG islands. Total CpG site refers to the entire CpG site probes that are for both CpG islands, including sites at the north and south shores and shelves, and non-CpG islands. There was a statistically significant difference in genome-wide DNA methylation between cases and controls for total CpG sites, and when separately analysing CpG island and non-CpG island methylation. A Wilcoxon Signed-Rank Sum test was used for total and non-CpG island methylation due to its skewed distribution. We found a mean difference of \(-0.0059 \pm 0.0147\) (\(p = 0.013\)) for the total 804,449 CpG sites and \(-0.0078 \pm 0.0147\) (\(p = 0.015\)) for non-CpG islands only. Significance was also found for CpG islands using a Student’s paired T-test with a mean difference of \(-0.0036 \pm 0.0069\) (\(p = 0.022\)) (Table 4.2.2.1).

**Table 4.2.2.1. Genome-wide methylation mean \(\beta\)-value difference between cases and control for the total, CpG island and non-CpG island based on a paired T-test and Wilcoxon Signed-Rank Sum test**

<table>
<thead>
<tr>
<th></th>
<th>Mean (\beta)-value Difference (n=22)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total (804,449)</strong></td>
<td>-0.0059(\pm)0.0147</td>
<td>0.013*</td>
</tr>
<tr>
<td><strong>CpG Island (351,400)</strong></td>
<td>-0.0036(\pm)0.0069</td>
<td>0.022*</td>
</tr>
<tr>
<td><strong>Non-CpG Island (453,049)</strong></td>
<td>-0.0078(\pm)0.0147</td>
<td>0.015*</td>
</tr>
</tbody>
</table>

*\(p\)-value based on a Wilcoxon Signed-Rank Sum test due to skewed distribution.
*\(p\)-value based on the paired-T test.

4.2.3 Gene-Specific Methylation

4.2.3.1 Genes differentially methylated between the cases and controls in cord blood MNCs

Quilcure Omics Explorer was used to calculate the largest difference between the cases and controls, and a paired T-test was used to compare difference between the groups. 98 CpG sites and 66 CpG-associated genes (Table 4.2.3.1a) were identified that were differentially methylated with a \(p\)-value of 0.007. A Benjamini-Hochberg correction for multiple testing was applied to each CpG site (804,449 sites) resulting in a \(q\)-value of 0.999.
DNA methylation patterns of $\beta$-values for CpG sites and their associated genes ‘CpG-site-associated-genes’ for the cases and controls are illustrated using an unsupervised hierarchical clustering approach, using the Euclidean distance method for clustering, and a heat map (Figure 4.2.3.1a). The heat map revealed distinctively different DNA methylation profiles between the cases (dark green) and controls (light green). Cases are shown to be hypomethylated (blue squares) in 75% of the CpG sites. Infant anthropometric measures were compared using a cutoff based on the mean of the 44 subjects, where 1 indicates below the mean and 2 indicates above the mean. Infant sex, infant gestational age and chip are distributed randomly between cases and controls indicating no sex, gestational age and batch effect between groups. Head circumference (‘HC’), birth weight (‘BW’), and birth weight percentile (‘BW%ile’) are shown to be greater in the cases than in the controls (Figure 4.2.3.1a). Although, borderline significance was only observed for birth weight percentile based on a Wilcoxon Rank-Sum test. Birth length (‘BL’) did not show patterns in distribution in either the case or control groups.

### Table 4.2.3.1a. Summary of the number of genes differentially methylated in cord blood MNCs relative to the control based on a T-test

<table>
<thead>
<tr>
<th>T-test</th>
<th>Hypermethylated</th>
<th>Hypomethylated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(p=0.007 \ q=0.999)$</td>
<td>21 (0.08%)</td>
<td>45 (0.17%)</td>
<td>66 (0.25%)</td>
</tr>
</tbody>
</table>

The numbers in brackets indicate the percentage of genes differentially methylated relative to total genes (25,159) targeted in the Illumina Infinium MethylationEPIC Array.
Figure 4.2.3.1a. Unsupervised hierarchical clustering and heat map of DNA methylation β-value in the case and control groups. Each row is a CpG site and each column is either a case or a control subject. Groups in dark green are the cases and light green are the controls. Blue squares within the heat map indicate hypomethylation and yellow squares indicate hypermethylation. For infant sex, males are light blue and females are burgundy. For head circumference, pale green indicates below the cutoff, orange is above, and white indicates missing. For birth weight, magenta indicates below the cutoff and light purple for above. For birth weight percentile, sky blue indicated below the cutoff and dark orange for above. Subject identification is shown in the bottom axis and CpG-site-associated-genes are shown in vertical axis.
In addition to the T-test, a Mann-Whitney U-test (MWU) was used to filter the most significant and largest magnitude CpG sites based on diffscore and $\Delta\beta$ values, respectively. 912 CpG sites were identified with 552 sites hypomethylated and 360 hypermethylated. 381 genes were associated with CpG sites with 219 genes hypomethylated and 162 genes hypermethylated (Table 4.2.3.1b).

**Table 4.2.3.1b. Summary of the number of genes differentially methylated in cord blood MNCs relative to the control based on a Mann-Whitney U-test**

<table>
<thead>
<tr>
<th>Mann-Whitney U-test</th>
<th>Hypermethylated</th>
<th>Hypomethylated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>(DiffScore = $&gt;</td>
<td>13</td>
<td>$)</td>
<td>162 (0.6%)</td>
</tr>
<tr>
<td>$\Delta\beta =</td>
<td>&gt;0.1</td>
<td>$</td>
<td></td>
</tr>
</tbody>
</table>

A principal component analysis (PCA) plot was used to identify the largest variation in the data. The first principal component (PCA 1) explains most of the variation, the second (PCA 2) or any other subsequent principal components accounts for any remaining variation. Our PCA analysis demonstrated that 23% of the variation in the data is explained by the difference in $B_{12}$ status of cases and controls (Figure 4.2.3.1c). PCA 1 (x-axis) represented the largest variation in the data where the cases (dark green) and controls (light green) are dispersed in two clusters. PCA 2 (y-axis) represented 6% of the variation in the data. These can be due to other environmental factors such as maternal or infant characteristics.
Figure 4.2.3.1c. **Principal component analysis plot of DNA methylation.** PCA plot indicates the largest variation clusters in the data of $\beta$-values for 804,449 CpG sites. Points represent all 44 subjects where blue dots are the cases and yellow dots are the controls. PCA 1 is the x-axis and PCA 2 is the y-axis.

4.2.3.2 Functional analysis on differentially methylated genes of the cases and controls in cord blood MNCs

Functional analysis on the 66 (T-test-generated), 381 (total MWU test-generated), 219 (hypomethylated MWU-test-generated), and 162 (hypermethylated MWU-test-generated) genes were performed using IPA to identify disease, molecular, cellular and physiological processes most relevant to the differentially methylated genes. Some genes were assigned to more than one category. These four datasets were generated, as mentioned in Table 4.2.3.1a and b, using the T-test and the MWU test. The differences between the three MWU-test-generated datasets is in that functional analysis was performed in the total number of genes (381), and separately as hypomethylated (219) and hypermethylated (162) genes in order to better elucidate functional processes specific to gene methylation. The top five molecular, cellular, physiological functions and disease processes for the 66 genes are presented based on significance. Cell and molecular functions were associated with genes in metabolism, cell cycle, molecular transport and small
molecule biochemistry. Genes related to behaviour, endocrine and nervous system, and embryonic and
hair and skin development were physiological system development and functions. Diseases and disorders
were associated with genes in cancer, neurological, cardiovascular, hematological diseases and organismal
abnormalities (Table 4.2.3.2a).

Table 4.2.3.2a. The top molecular, cellular, physiological functions and disease processes for
the 66 hyper- and hypomethylated genes

<table>
<thead>
<tr>
<th>Category</th>
<th>p-value range</th>
<th>No. of Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular and Cellular Functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>$2.22 \times 10^{-4}$</td>
<td>3</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>$3.47 \times 10^{-4}$</td>
<td>7</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>$2.53 \times 10^{-4}$</td>
<td>11</td>
</tr>
<tr>
<td>Small molecule biochemistry</td>
<td>$3.47 \times 10^{-4}$</td>
<td>11</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>$2.84 \times 10^{-4}$</td>
<td>4</td>
</tr>
<tr>
<td><strong>Physiological System Development and Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behaviour</td>
<td>$2.84 \times 10^{-4}$</td>
<td>3</td>
</tr>
<tr>
<td>Embryonic</td>
<td>$4.39 \times 10^{-4}$</td>
<td>5</td>
</tr>
<tr>
<td>Endocrine</td>
<td>$3.20 \times 10^{-4}$</td>
<td>1</td>
</tr>
<tr>
<td>Hair and Skin</td>
<td>$2.84 \times 10^{-4}$</td>
<td>2</td>
</tr>
<tr>
<td>Nervous system</td>
<td>$4.39 \times 10^{-4}$</td>
<td>3</td>
</tr>
<tr>
<td><strong>Diseases and Disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>$4.60 \times 10^{-4}$</td>
<td>59</td>
</tr>
<tr>
<td>Neurological</td>
<td>$4.39 \times 10^{-4}$</td>
<td>24</td>
</tr>
<tr>
<td>Organismal Injury and Abnormalities</td>
<td>$4.60 \times 10^{-4}$</td>
<td>60</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>$4.39 \times 10^{-4}$</td>
<td>8</td>
</tr>
<tr>
<td>Hematological</td>
<td>$4.70 \times 10^{-4}$</td>
<td>19</td>
</tr>
</tbody>
</table>

Five networks were identified and ranked by a network score in the p-value calculation of the IPA, which
ranged from 2 to 63. The highest-scoring network of 63 revealed a significant link with cell death and
survival, cellular development, cellular growth, and proliferation. The remaining three networks had a
score of 2 and were linked to cancer, hematological, immunological, cardiovascular disease, cellular
assembly and organization, DNA replication, recombination and repair, and cell signalling. The network
score indicates the likelihood of the Focus Genes in a network being found together due to random chance.
The Focus Genes indicate the uploaded genes of interest for which information is available in the Ingenuity
Knowledge Base.
The top five molecular, cellular, physiological functions and disease processes for the 219 hypomethylated and 162 hypermethylated genes are presented based on significance. Common cell and molecular functions are associated with genes in cell cycle. Physiological system development and functions are associated with genes in connective tissue development. Diseases and disorders are associated with genes in cancer, organismal abnormalities, gastrointestinal disease and endocrine system disorders (Table 4.3.2b).

Table 4.2.3.2b. The top molecular, cellular, physiological functions and disease processes for the 219 hypomethylated and 162 hypermethylated genes

<table>
<thead>
<tr>
<th>Category</th>
<th>Hypomethylated</th>
<th></th>
<th>Category</th>
<th>Hypomethylated</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value range</td>
<td>No. of Genes</td>
<td></td>
<td>p-value range</td>
<td>No. of Genes</td>
</tr>
<tr>
<td><strong>Molecular and Cellular Functions</strong></td>
<td></td>
<td></td>
<td><strong>Molecular and Cellular Functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular movement</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 2.75&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>32</td>
<td>Cellular growth and proliferation</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 2.95&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>24</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 2.75&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>11</td>
<td>Cell cycle</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 1.70&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 2.75&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>9</td>
<td>Cell death and survival</td>
<td>4.23&lt;sup&gt;-2&lt;/sup&gt; – 2.15&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>18</td>
</tr>
<tr>
<td>Small molecule biochemistry</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 2.75&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>21</td>
<td>Gene expression</td>
<td>7.07&lt;sup&gt;-2&lt;/sup&gt; – 4.47&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 4.42&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>17</td>
<td>Cellular development</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 4.70&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>23</td>
</tr>
<tr>
<td><strong>Physiological System Development and Function</strong></td>
<td></td>
<td></td>
<td><strong>Physiological System Development and Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>4.06&lt;sup&gt;-2&lt;/sup&gt; – 2.47&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>17</td>
<td>Hematological</td>
<td>4.52&lt;sup&gt;-2&lt;/sup&gt; – 2.95&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>3.83&lt;sup&gt;-2&lt;/sup&gt; – 4.86&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>6</td>
<td>Lymphoid</td>
<td>2.38&lt;sup&gt;-2&lt;/sup&gt; – 4.70&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>Skeletal and Muscular</td>
<td>3.83&lt;sup&gt;-2&lt;/sup&gt; – 4.86&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>4</td>
<td>Reproductive</td>
<td>3.49&lt;sup&gt;-2&lt;/sup&gt; – 5.58&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 5.05&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>16</td>
<td>Immune cell trafficking</td>
<td>4.52&lt;sup&gt;-2&lt;/sup&gt; – 7.05&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>Organismal</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 5.05&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>19</td>
<td>Connective tissue</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 7.07&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td><strong>Diseases and Disorders</strong></td>
<td></td>
<td></td>
<td><strong>Diseases and Disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>4.72&lt;sup&gt;-2&lt;/sup&gt; – 1.60&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>176</td>
<td>Cancer</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 6.56&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>122</td>
</tr>
<tr>
<td>Organismal Injury and Abnormalities</td>
<td>4.76&lt;sup&gt;-2&lt;/sup&gt; – 1.60&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>180</td>
<td>Organismal Injury and Abnormalities</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 6.56&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>126</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>4.66&lt;sup&gt;-2&lt;/sup&gt; – 1.92&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>156</td>
<td>Endocrine</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 9.26&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>66</td>
</tr>
<tr>
<td>Hepatic</td>
<td>4.66&lt;sup&gt;-2&lt;/sup&gt; – 1.92&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>94</td>
<td>Gastrointestinal</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 9.26&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>116</td>
</tr>
<tr>
<td>Endocrine</td>
<td>4.72&lt;sup&gt;-2&lt;/sup&gt; – 1.10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>74</td>
<td>Metabolic</td>
<td>4.85&lt;sup&gt;-2&lt;/sup&gt; – 9.26&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>28</td>
</tr>
</tbody>
</table>
Five networks were identified and were ranked by a network score in the $p$-value calculation of the IPA, which ranged from 2 to 67 for hypomethylated genes and 60 for hypermethylated genes. The highest-scoring networks for hypomethylated genes of 67 and 65 are linked with cell death and survival, cellular development, growth and proliferation, and cellular movement. The remaining three networks have a score of 17 and 2 and show similar related links in addition to organismal injury and abnormalities, free radical scavenging, and molecular transport. For hypermethylated genes, network scores of 60 and 56 are linked with organismal, cellular growth and proliferation, gene expression, cell-to-cell signaling and interaction, and cardiovascular system development and function. Lower-ranking network scores of 2 are linked to cellular assembly and organisation, DNA replication, recombination, repair, cell cycle, cancer, cell death and survival and morphology. Summary table of IPA functions for common genes for all three gene groups, including 371 total MWU-test functional analysis, are shown in Appendix D.

Common genes between generated by the T-test and MWU-test are illustrated using a Venn diagram (Figure 4.2.3.2). 27 genes overlap where 15 are hypomethylated and 12 are hypermethylated in the case group.

![Venn diagram showing gene overlap](image)

**Figure 4.2.3.2.** Number of genes generated by T-test and MWU-test that are commonly differentially methylated in cord blood MNCs.
4.2.4 Gene Expression

4.2.4.1 Candidate genes selected for gene expression analysis

A total of 14 genes were selected for gene expression analysis, of which 7 are hypo- and 7 are hypermethylated relative to the controls (Table 4.2.4.1). Genes were selected from the 27 overlap genes common to both the T-test and MWU-test generated genes. The most hypo- and hypermethylated genes based on fold change were selected. Genes were also chosen depending on functional relevance to growth, development and metabolism based on IPA analyses. Gene expression in cord MNCs was estimated from lymphocytes, monocytes and B cells in peripheral blood using BioGPS database (http://biogps.org). This was assessed for all candidate genes to determine whether the genes were expressed in MNCs and in what level, so that we can estimate what gene expression level we can expect for ddPCR.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Names</th>
<th>Top Biological Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypomethylated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PPAP2B</strong></td>
<td>Lipid phosphate phosphohydrolase 3</td>
<td>biosynthetic process; nitrogen and phospholipid compound metabolic processes; regulation of biological process; response to stimulus; signal transduction</td>
</tr>
<tr>
<td><strong>DRD4</strong></td>
<td>D(4) dopamine receptor</td>
<td>heterotrimeric G-protein signalling pathway; nicotine pharmacodynamics pathway; dopamine receptor mediated signalling pathway</td>
</tr>
<tr>
<td><strong>SCD</strong></td>
<td>Acyl-CoA desaturase</td>
<td>gene expression regulation; lipogenesis; regulation of mitochondrial fatty acid oxidation; energy homeostasis; biosynthesis of membrane phospholipids; cholesterol esters and triglycerides</td>
</tr>
<tr>
<td><strong>ARHGEF12</strong></td>
<td>Rho guanine nucleotide exchange factor 12</td>
<td>protein binding; small GTPase regulator activity; guanyl-nucleotide exchange factor activity</td>
</tr>
<tr>
<td><strong>SLC25A24</strong></td>
<td>Calcium-binding mitochondrial carrier protein</td>
<td>biosynthetic and cellular process; translation; amino acid transporter</td>
</tr>
<tr>
<td><strong>PRKCH</strong></td>
<td>Protein kinase C eta type</td>
<td>intracellular signal transduction; phosphate-containing compound metabolic process; regulation of biological process; response to stimulus; pathways related to cardiovascular, nervous systems and growth</td>
</tr>
<tr>
<td><strong>APBB2</strong></td>
<td>Amyloid beta A4 precursors protein-binding family B member 2</td>
<td>biosynthetic and cellular process; nitrogen compound metabolic process; regulation of nucleobase-containing compound metabolic process; DNA-dependent transcription</td>
</tr>
<tr>
<td><strong>HLA-E</strong></td>
<td>HLA class I histocompatibility antigen, alpha chain E</td>
<td>antigen processing and presentation; major histocompatibility complex antigen</td>
</tr>
<tr>
<td><strong>LIF</strong></td>
<td>Leukemia inhibitory factor</td>
<td>antigen processing and presentation</td>
</tr>
<tr>
<td><strong>FRMD4B</strong></td>
<td>FERM domain-containing protein</td>
<td>cellular component morphogenesis and process</td>
</tr>
<tr>
<td><strong>MYT1L</strong></td>
<td>Myelin transcription factor 1-like protein</td>
<td>regulation of transcription from RNA polymerase II promoter</td>
</tr>
<tr>
<td><strong>CYFIP1</strong></td>
<td>Cytoplasmic FMR1-interacting protein 1</td>
<td>signal transduction; Huntington disease pathway (tumour protein p53)</td>
</tr>
<tr>
<td><strong>GRIN2A</strong></td>
<td>Glutamate receptor ionotrophic, mitochondrial</td>
<td>signal transduction; Huntington disease pathways (ionotropic and metabotropic glutamate receptor pathways)</td>
</tr>
<tr>
<td><strong>DOCK10</strong></td>
<td>Deducator of cytokinesis protein 10</td>
<td>cellular component movement; intracellular protein transport and transduction; phagocytosis</td>
</tr>
</tbody>
</table>

Gene names and biological functions were obtained from PANTHER database.
4.2.4.2 Gene expression analysis based on ddPCR

All genes showing expression are based on a mean copy number difference that is relative to the control. Nine out of 14 genes showed an increase in gene expression regardless of whether the DNA methylation status was hypo- or hypermethylated. Three hypomethylated genes (PPAP2B, DRD4 and SCD) and two hypermethylated genes (GRIN2A and DOCK10) showed decreased gene expression. ARHGEF12 is the only gene shown to have a statistically significant increase in gene expression with a mean copy difference of 169.5 ± 54.1 (p=0.008) based on a paired T-test (Table 4.2.4.2). Bar graphs are shown in Figures 4.2.4.2a,b,c as a visual representation of the changes in gene expression per gene and their variation.

ARHGEF12 was the second most highly expressed gene in cord blood MNCs, after HLA-E. Generally, our selected genes are not highly expressed in cord blood MNCs. Nevertheless, in comparison with each other, 7 genes show a greater level of expression while the other half show a lower level of gene expression regardless of DNA methylation status.

Table 4.2.4.2. ddPCR gene expression analysis for candidate genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene expression (n=4)</th>
<th>Mean copy difference (± stdev)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hypomethylated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPAP2B</td>
<td>↓</td>
<td>-3.57 ± 8.75</td>
<td>p=0.474</td>
</tr>
<tr>
<td>DRD4</td>
<td>↓</td>
<td>-0.07 ± 1.11</td>
<td>p=0.914</td>
</tr>
<tr>
<td>SCD</td>
<td>↓</td>
<td>-2.05 ± 6.62</td>
<td>p=0.580</td>
</tr>
<tr>
<td>ARHGEF12*</td>
<td>↑</td>
<td>169.5 ± 54.1</td>
<td>p=0.008</td>
</tr>
<tr>
<td>SLC25A24</td>
<td>↑</td>
<td>28.0 ± 40.6</td>
<td>p=0.262</td>
</tr>
<tr>
<td>PRKCH</td>
<td>↑</td>
<td>103.5 ± 67.0</td>
<td>p=0.054</td>
</tr>
<tr>
<td>APBB2</td>
<td>↑</td>
<td>0.18 ± 0.95</td>
<td>p=0.738</td>
</tr>
<tr>
<td><strong>Hypermethylated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-E</td>
<td>↑</td>
<td>755.5 ± 553.1</td>
<td>p=0.072</td>
</tr>
<tr>
<td>LIF</td>
<td>↑</td>
<td>0.38 ± 0.43</td>
<td>p=0.433</td>
</tr>
<tr>
<td>FRMD4B</td>
<td>↑</td>
<td>2.9 ± 13.3</td>
<td>p=0.693</td>
</tr>
<tr>
<td>MYT1L</td>
<td>↑</td>
<td>0.27 ± 0.19</td>
<td>p=0.063</td>
</tr>
<tr>
<td>CYFIP1</td>
<td>↑</td>
<td>17.1 ± 15.9</td>
<td>p=0.121</td>
</tr>
<tr>
<td>GRIN2A</td>
<td>↓</td>
<td>-0.34 ± 0.50</td>
<td>p=0.275</td>
</tr>
<tr>
<td>DOCK10</td>
<td>↓</td>
<td>-3.88 ± 43.8</td>
<td>p=0.8709</td>
</tr>
</tbody>
</table>

* indicates significance (p<0.05)
Figure 4.2.4.2a. Gene expression on candidate gene mean copy number. Absolute measurement of mRNA expression shown as mean copy number (± standard deviation) between cases and controls. Bars represent average copy number with standard deviation. Asterisk (*) represents significant differences between cases and controls (n=4). Significance was determined if p<0.05.

ARGEF12, PRKCH and SLC25A24 for hypomethylated and HLA-E then DOCK10, CYFIP1 and FRMD4B for hypermethylated genes show a greater level of gene expression in both cases and controls (Figure 4.2.4.2b).
Figure 4.2.4.2b. Gene expression of high expressing hypo- and hypermethylated genes.
Absolute measurement of mRNA expression of cases and controls. Bars represent average copy number with standard deviation. Asterisks (*) represent significant differences between cases and controls (n=4). Significance was determined if p<0.05.
*PPAP2B, DRD4, SCD,* and *APBB2* for hypomethylated and *LIF, MYT1L* and *GRIN2A* for hypermethylated genes show a lower level of gene expression in cases and controls (**Figure 4.2.4.2c**).

**Figure 4.2.4.2c. Gene expression of low expressing hypo- and hypermethylated genes.**

Absolute measurement of mRNA expression of cases and controls. Bars represent average copy number with standard deviation.
4.3 Discussion

In the present study, we explored 1) genome-wide and 2) gene-specific DNA methylation in cord blood MNCs comparing infants born to mothers with low B12 status with those of infants born to mothers with high B12 status in a high folate environment. We further investigated 3) functional, biological and clinical ramifications associated with the observed DNA methylation changes. Our exploratory findings are discussed below.

4.3.1 Lower genome-wide DNA methylation in infants born to mothers with low B12 status

Infants born to mothers with low B12 status in a high folate environment during early pregnancy were found to have significantly lower genome-wide DNA methylation (total CpG, CpG islands and non-CpG islands sites) in cord blood MNCs compared to infants born to mothers with high B12 status (Table 4.3.2.1). The observed lower genome-wide DNA methylation in cord blood MNCs in infants born to mothers with low B12 status is likely related to the methyl trap. As B12 functions as a coenzyme to MS, inadequate B12 levels would reduce MS activity, which catalyses the transfer of a methyl group from 5-MTHF to remethylate homocysteine to methionine, resulting in folate that is ‘trapped’ in the form of 5-MTHF. This results in lower levels of SAM, the universal methyl group donor to DNA, proteins, lipids and metabolites for most biological reactions including DNA methylation, and thus lower DNA methylation52 (Figure 2.1.3). Animal studies have more consistently shown a reduction of DNA methylation with B12 deficiency117,355–357.

With high RBC folate concentrations, hypomethylation may also be observed from two potential mechanisms. Firstly, the observed high RBC folate status attributable to prenatal folic acid supplementation can lead to excess DHF, which inhibits MTHFR, and thereby reduces 5-MTHF. Since 5-MTHF donates its methyl to SAM for DNA methylation, a reduction would subsequently lead to DNA hypomethylation158. Secondly, preferential shuttling from the DNA methylation pathway to the DNA synthesis pathway
through upregulation of TS\textsuperscript{292} may occur with high folate concentrations. Folic acid has been shown to
upregulate DHFR in certain situations\textsuperscript{359}, and therefore also upregulate TS activity as transcription of both
DHFR and TS genes are coregulated\textsuperscript{360}. As a result, an increase in thymidylate may be observed at the
expense of DNA methylation\textsuperscript{361} (Figure 2.1.3). Hypomethylation has been associated with folic acid
supplementation in animal studies\textsuperscript{291–293}, albeit inconsistently, and can be attributed to the varying folate
concentrations, duration of study and methods of assessment utilized in animal studies.

In contrast to our findings, a human study looking at the effects of RBC folate and serum B\textsubscript{12} found that
increased maternal B\textsubscript{12} during pregnancy was associated with decreased genome-wide DNA methylation in
newborns\textsuperscript{334}. Reasons for this may be due to the differences in serum B\textsubscript{12} and RBC folate concentrations
between our studies. The McKay et al. 2012\textsuperscript{334} study correlated serum B\textsubscript{12} with DNA methylation based on
a higher mean B\textsubscript{12} concentration of 209 (226, 389) pmol/L compared to our study’s low B\textsubscript{12} group of 120
(112, 128) pmol/L, as well as the <148 pmol/L deficiency cutoff for serum B\textsubscript{12}. It may be that the B\textsubscript{12}
concentration they reported is too high to have decreased MS and SAM activity, and thereby also decrease
methylation. Their study also reported RBC folate concentration to be 859 (675, 1160) nmol/L, which is
approximately three times lower than our study’s folate concentration for the low B\textsubscript{12} group of 2682
(2506, 2858) nmol/L, and is also below the 1000 nmol/L cutoff for maximal protection against NTDs.
This concentration may not have been high enough to have increased DHF and reduce MTHFR activity.
Similarly, preferential shuttling to the DNA synthesis pathway may also not have occurred.

One of the major findings of the PREFORM study showed similar findings with ours. Their study found
that lower genome-wide DNA methylation was observed in cord blood MNCs in infants born to mothers
with high RBC folate (≥2860 nmol/L) and low serum B\textsubscript{12} (≤167 pmol/L) concentrations than infants born
to mothers with lower RBC and higher serum B\textsubscript{12} concentrations\textsuperscript{33}. We observed very high RBC folate
concentrations and lower serum B\textsubscript{12} concentrations in the PREFORM study compared to McKay et al.
The PREFORM study also had similar B₁₂ and folate concentrations to the present study. Hence, a higher folate and lower B₁₂ concentration may be needed to see a decrease in genome-wide DNA methylation. In addition to the actual supplemental levels of B₁₂ and folate, the ratio of B₁₂ to folate may be also important in modulating genome-wide DNA methylation. In a rat model study, where maternal folic acid supplementation (4X the basal requirement) was studied with or without B₁₂ on placental genome-wide DNA methylation, a decrease in genome-wide DNA methylation in the absence of B₁₂ was observed. This suggests that the ratio of folic acid and B₁₂, in addition to the actual supplemental levels, may have an important role in determining genome-wide DNA methylation patterns. In our study and in the PREFORM study, our B₁₂ to folate ratio and our genome-wide DNA methylation findings resemble the findings in the rat study. Although our sample population may not be fully representative of the Canadian population, our findings provide relevant insight to our Canadian WCBA population who have been shown to have high concentrations of RBC folate, and a proportion of them with suboptimal B₁₂ status despite adequate intakes. Hence, both the supplemental levels and the ratio of B₁₂ to folate highlight the importance of the current recommendations for B₁₂ and folate, and the potential need to change these recommendations in order to provide adequate B₁₂ throughout each trimester and perhaps a more conservative dose recommendation for prenatal folic acid supplementation.

4.3.2 Differentially regulated genes are more hypomethylated in mothers with low B₁₂ status

On a gene-specific DNA methylation scale, we observed findings similar to the genome-wide DNA methylation data. We interrogated DNA methylation status of 804,449 individual CpG sites of 25,158 genes using the Illumina Infinium MethylationEPIC array. Cord blood MNC DNA from infants born to mothers with low B₁₂ had more hypomethylated genes (p=0.007, q=0.999) than hypermethylated genes, 45 and 21, respectively. Significance based on the T-test p-value was observed without bias of batch number, infant sex and infant gestational age. However, multiple comparison testing did not reach statistical significance. Similar findings, in that there were a greater number
of hypo- than hypermethylated genes, were observed with genes obtained from the non-parametric MWU-test (219 hypo- and 162 hypermethylated) (Table 4.2.1b) and overlapping genes (15 hypo- and 12 hypermethylated) (Figure 4.2.3.2). To address the extent of how DNA methylation alterations between the cases and controls differed, we performed PCA according to the DNA methylation status of the interrogated CpG sites and its associated genes. A two-dimensional PCA plot detected two distinct variations of 23% and 6%. PCA 1 with 23% of the variation is explained by the differences in maternal B<sub>12</sub> status of the cases and controls in the presence of high folate status (Figure 4.2.3.1c). A change in methylation ≥5% has been considered biologically significant<sup>364,365</sup>.

4.3.3 Ramifications of B<sub>12</sub> and folate induced changes in DNA methylation on functional and clinical significance of hypo- and hypermethylated genes

We also performed functional analyses using IPA in order to elucidate the functional ramifications of the hypo- and hypermethylated genes that differed most between the cases and controls. Functional analyses revealed several molecular and cellular functions, including macromolecule metabolism and cell cycle, were affected by changes in B<sub>12</sub> and folate. This finding in particular was observed for genes that were hypomethylated. Hypermethylated genes were associated with cellular growth, proliferation, death and survival, development, and gene expression (Table 4.3.4). Additionally, functional analyses provided insight into associated physiological system development and functions as well as diseases and disorders for the hypo- and hypermethylated genes that were influenced by maternal B<sub>12</sub> status in a high folate environment. These genes are shown to be involved in organ and organismal development, in diseases related to cancer and organismal injury and abnormalities, and metabolic disorders (Table 4.3.4). Although exploratory and preliminary, our functional analyses provided insight into the molecular, cellular, and physiological processes potentially involved in metabolic diseases such as obesity, type 2 diabetes, and metabolic syndrome.
From our clinical anthropometric measures, we found a trend towards a higher birth weight, birth weight percentile, and larger head circumference in infants born to mothers with low B₁₂ status in a high folate environment compared with those born to mothers with high B₁₂ status based on the 66 differentially methylated genes generated by the T-test (Figure 4.3.1a). While the small sample size and subsequent lack of power limited our study, birth weight percentile showed borderline significance (p=0.055) based on a Wilcoxon Rank-Sum test (Table 4.2.1.2d). However, this finding may lose its significance using the z-score method for calculating birth weight percentile. Nevertheless, our observed trend towards a larger-sized infant from mothers with low B₁₂ status amidst a high folate environment supports the finding of the PREFORM study where infants born to mothers with high RBC folate and low serum B₁₂ concentrations had a higher birth weight (3697 ± 455 g) in comparison to infants born to mothers with lower RBC and higher serum B₁₂ concentrations (3393 ± 451 g) (p=0.001). Furthermore, despite the lack of statistical significance observed for an increase in infant birth weight, it is possible that changes in body weight may be observed after birth. Animal studies have shown that although no initial change in birth weight was observed in the offspring, dams fed a high multivitamin supplement (including folic acid) had male and female pups that had a higher post-weaning body weight than controls.

Studies solely investigating B₁₂, associated a low B₁₂ status with a lower birth weight and a higher risk for SGA. Similarly, low B₁₂ intakes in the presence of high total folate intake was associated with low birth weight and higher risk for SGA. Smaller birth length, head and chest circumference were also observed with a high plasma folate to B₁₂ ratio. Although these studies may differ from our findings, our studies varied due to the differences in nutrition status of the populations of the studies, infant mean birth weight, and measurement of B₁₂ and folate concentrations. The studies focusing on B₁₂ alone, and in combination with folate, were based on mothers who were predominantly vegetarian, and were reported to have inadequate intakes of dietary B₁₂. Additionally, plant-based diets are known to be low in other nutrients such as vitamin D and zinc, which have been associated with low birth weight. The
inadequacy of serum $B_{12}$ in our study was not due to insufficient intakes, as mothers had dietary intakes above the EAR and most supplemented with a form of $B_{12}$-containing vitamin, but can rather be attributed to the demands of the infant during pregnancy$^{14,206}$. This dissimilarity in diet may also contribute to the differences seen between our study’s infant anthropometry. The birth weights between our study’s may also not be comparable as our infant birth weight was larger by approximately 1000 g. In addition, these studies, except for Muthayya et al. 2006$^{226}$, measured either plasma folate and $B_{12}$ or only intakes$^{12,368}$. Finally, while our study focused on early pregnancy $B_{12}$ and folate concentrations, other studies reported measurements at different periods during the pregnancy.

Although it has been shown that $B_{12}$ and birth weight are positively associated, folate has been shown to be even more consistently associated with birth weight$^{115,116,165,169}$, and negatively with risk for SGA$^{167}$ and IUGR$^{368}$. Since our study focused on a combination with high folate status, it is possible that folate drives changes in birth weight. Moreover, similar to the effect on DNA methylation as previously described, the ratio of $B_{12}$ to folate may be important in affecting infant birth weight. An observational study found that the ratio of folate to $B_{12}$ was associated with neonatal anthropometry, whereas maternal plasma folate and $B_{12}$ concentrations were not$^{362}$. 
4.3.4 Upregulation of B12 and folate induced hyper- and hypomethylated genes

Based on functional relevance and the greatest change in DNA methylation and p-value significance of genes generated by the T-test, MWU-test and its overlap, 14 genes were selected for gene expression analysis. Functional relevance of the selected genes was primarily associated with lipid metabolism, molecular transport and small molecule biochemistry. This association was more evident in the hypomethylated genes.

ddPCR was utilized to provide absolute measurement of gene expression data without the need for a housekeeping gene and could better detect changes in low expression levels. Although we did not find statistical significance between the mean copy differences except for ARHGEF12 (169.5 ± 54.1 (p=0.008)), this is likely due to our small sample size, and can also be attributed to the fact that gene expression levels vary across different tissues. Even if DNA methylation changes occur across all tissues, gene expression changes may occur in specific tissues. We only examined gene expression in cord blood MNCs in the present study. Nevertheless, an increase in gene expression was observed in 9 out of 14 genes (Table 4.2). This can be expected as hypomethylated CpG sites often allow for transcription to occur due to its effect on transcription machinery. A study investigating B12 deficiency reported upregulation of hypomethylated genes of cholesterol biosynthesis regulators in women. Another study based on a pig model found that methylating micronutrient supplementation showed differentially expressed metabolic genes after a change in methylation. This study also reported phenotypic changes associated with decreased back fat percentage, adipose tissue, fat thickness and increased shoulder percentage in the F2 generation.

ARHGEF12 particularly has been associated with myeloid leukemia, and shown to be expressed in prostate cancer cell lines and squamous cell carcinoma (HSC-3) cells. Interestingly, leukemia-associated ARHGEF12 has been shown to increase cell growth when it is in complex with CD44, a cell-surface glycoprotein, and EGFR, an epidermal growth factor, in HSC-3 cells. Upregulation of ARHGEF12 may thus be potentially involved with infant growth and development, and with the observed increase in
infant anthropometric measures. Another gene that is upregulated is \textit{FRMD4B}, which has been shown to play a critical role in insulin receptor signaling\textsuperscript{374}. Similarly, \textit{SCD} was shown to be involved in diet-induced hepatic insulin resistance, where downregulation of \textit{SCD} decreased glucose production by 75\%, and decrease both gluconeogenesis and glycogenolysis via reduced activity of glucose-6-phosphotase and phosphoenolpyruvate carboxykinase\textsuperscript{375}. \textit{SCD} has also been inversely associated with hypertriglyceridemia in mice and humans through impaired triglyceride and cholesterol biosynthesis\textsuperscript{376}. The relevance of these genes to clinical measures related to metabolic disease highlights the need of these B12 and folate induced DNA methylated genes to potentially influence infant health and disease outcomes at birth and later in life.

Although gene expression analysis was limited by RNA quality, expression in MNCs and sample size, our findings provide potentially interesting information regarding the influence of B12 and folate induced changes in DNA methylation on gene expression regulation. Future studies are needed to confirm the aforementioned associations and to further explore the effect of B12 and folate on DNA methylation and gene expression.

### Table 4.3.4. Summary table of gene expression and functional analyses of hypo- and hypermethylated genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Methylation status</th>
<th>Gene expression</th>
<th>Top Molecular/Cellular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{PPAP2B}</td>
<td>↓</td>
<td>↓</td>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
<td>\textit{DRD4}</td>
<td>↓</td>
<td>↓</td>
<td>Lipid metabolism</td>
</tr>
<tr>
<td>\textit{SCD}</td>
<td>↓</td>
<td>↓</td>
<td>Molecular Transport</td>
</tr>
<tr>
<td>\textit{ARHGEF12}</td>
<td>↓</td>
<td>↑</td>
<td>Small Molecule Biochemistry</td>
</tr>
<tr>
<td>\textit{SLC25A24}</td>
<td>↓</td>
<td>↑</td>
<td>Behaviour</td>
</tr>
<tr>
<td>\textit{PRKCH}</td>
<td>↓</td>
<td>↑</td>
<td>Cell Cycle</td>
</tr>
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<td>\textit{APBB2}</td>
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<td>↑</td>
<td>Cellular movement</td>
</tr>
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<td>\textit{HLA-E}</td>
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<td>↑</td>
<td>Cell growth and proliferation</td>
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<td>↑</td>
<td>Cell death and survival</td>
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<td>\textit{FRMD4B}</td>
<td>↑</td>
<td>↑</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>\textit{MYT1L}</td>
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<td>↑</td>
<td>Cell development</td>
</tr>
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<td>Lipid metabolism</td>
</tr>
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<td>Molecular Transport</td>
</tr>
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</tr>
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<td></td>
<td>Behaviour</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gene Expression</td>
</tr>
</tbody>
</table>
4.4 Conclusion

The PREFORM study is a large observational study in a demographically diverse group of pregnant Canadian women and their infants. We explored whether or not changes in DNA methylation were influenced by maternal low serum B\textsubscript{12} and high RBC folate concentrations at early pregnancy. We also aimed to understand whether or not these B\textsubscript{12} and folate induced changes in DNA methylation have the potential to affect infant health and disease outcomes at birth and in adulthood. We thus examined functional relevance of differentially methylated genes, changes in gene expression, as well as in measurement of infant anthropometry (Figure 5.1).

We found that both genome-wide and gene-specific DNA methylation is reduced in cord blood MNCs of infants born to mothers with a low B\textsubscript{12} amidst a high folate environment. Genome-wide methylation analysis showed significance for mean $\beta$-value difference of the total (-0.0059±0.0147, $p=0.013$), CpG island only (-0.0036±0.0069, $p=0.022$), and non-CpG island only (-0.0078±0.0147, $p=0.015$) analyses. A total of 66 genes were found to be differentially methylated ($p=0.007$, $q=0.999$), 45 of which were hypomethylated and 21 relative to the control. A 23% difference in DNA methylation was observed between the cases and the control groups based on the PCA. Of the 14 selected genes, 9 showed upregulation and 5 showed downregulation regardless of DNA methylation status. *ARHGEF12* was shown to be significantly upregulated with a mean copy difference of 169.5 ± 54.1 ($p=0.008$) relative to the control. Functional analyses reveal that these genes are associated with metabolism and development. Infants born to mothers with a low B\textsubscript{12} status are shown to have a higher birth weight and birth weight percentile and a larger head circumference. Taken together, our exploratory findings support the notion that environmental nutrient exposure can be affected by epigenetic changes to potentially influence infant health and disease outcomes later in life, as described in the DOHaD hypothesis (Figure 5.1).
Future recommendations for $B_{12}$ and folate should be reconsidered in an attempt to lower RBC folate and raise serum $B_{12}$ concentrations in pregnant women. Future studies are warranted to confirm our findings with a larger sample size, and elucidate clinical ramifications on chronic disease risk-related factors such as adiposity and insulin resistance on children at 5 years of age and older.
5 - Overall Conclusions and Future Directions

5.1 Summary and General Discussion

The primary objective of this thesis was to determine whether or not maternal low B\textsubscript{12} and high folate status affects DNA methylation and gene expression in newborn infants’ cord blood MNCs. Based on the methyl trap theory, it was hypothesized that low maternal serum B\textsubscript{12} concentrations in combination with high folate status reduced genome-wide DNA methylation as well as gene-specific DNA methylation in genes that are related to fetal and infant growth and development (Figure 5.1).

![Diagram]

**Figure 5.1. Summary of thesis conclusion.** Similar to the DOHaD model, a tripartite relationship of maternal nutrient exposure, infant DNA methylation and possible future effects on chronic disease risk was observed.

We indeed demonstrated that infants born to mothers with low B\textsubscript{12} status amidst the presence of a high folate status had lower genome-wide DNA methylation and more hypomethylated genes compared with those born to mothers with higher B\textsubscript{12} status. Their molecular, physiological and functional relevance were shown to be associated with metabolism, organ and organismal development. We also found that these B\textsubscript{12} and folate induced hypomethylation was associated with upregulation of genes related to metabolism and
development in the newborn infant, which may lead to more pronounced phenotypic effects in early childhood. Our study provides novel information regarding the relationships between maternal B₁₂ and folate status at a critical period of development and DNA methylation. With currently high levels of folic acid in the Canadian WCBA population, and a proportion of pregnant women with suboptimal B₁₂ status despite adequate intakes, our findings are important in bringing to light possible changes in recommendations for supplementation during preconception and throughout each trimester. Although it is important to have recommendations that encourage preconceptional folic acid supplementation to prevent NTDs, previous studies including ours have shown high maternal RBC folate concentrations to have exceeded presently designated ‘high’ cutoffs. Their effects on infant health and disease have been shown to be adverse in relation to insulin resistance, adiposity, and risk for developing metabolic disorders; although, current evidence is inconclusive. B₁₂ dietary and supplemental intakes, on the other hand, appear to be at or exceeding the EAR of 2.6 µg/d for pregnant women. However, B₁₂ status has been shown to be suboptimal in some women; hence, modified recommendations for folate and B₁₂ should be considered.
5.2 Strengths and Limitations

To date, the PREFORM study is the largest Canadian observational study assessing maternal one-carbon nutrients including $B_{12}$ and folate concentrations on DNA methylation in their newborn infants\textsuperscript{143}. While the PREFORM original cohort is highly reflective of the multietnic population of Toronto, our findings, using a smaller subsample of matched cases and controls, may not represent all Canadians\textsuperscript{115}. Nonetheless, our data significantly adds to the growing body of evidence that suggests that maternal nutrition may have epigenetic effects in the offspring. Also, we are one of the few studies that measured the effects of $B_{12}$ and folate status on genome-wide and gene-specific DNA methylation in cord blood MNCs using the Illumina Infinium MethylationEPIC array. Another strength of our study is our exploratory analysis on elucidating biological ramifications of nutrient-induced epigenetic changes with functional and gene expression analyses using Ingenuity Pathway Analysis, ddPCR, and infant anthropometry. Our study is one of the few studies which investigated a tripartite relationship between nutrient status, DNA methylation and gene expression in relation to infant growth and development. The method of gene expression was also a strength of this study as ddPCR allowed for more accurate and reliable measurement of gene expression through absolute (without use of a housekeeping gene) instead of comparative analysis, as seen with RT-qPCR. In addition, we have amassed a large dataset of gene-specific DNA methylation patterns from cord blood MNCs that differ based on maternal $B_{12}$ status in a high folate environment, which can be used for future analyses.

There are a number of limitations to this study. The cases and controls were matched using propensity score analysis based on seven confounding variables that affect DNA methylation; however, the sample size was too small to correct other potential confounders in the regression. Hence, maternal ethnicity and smoking during pregnancy were not controlled for, among other residual confounders. For smoking during pregnancy, however, a small number of women smoked during pregnancy (5 out of 44) and were relatively balanced between cases and controls. Another limitation pertains to the cell type for DNA
methylation analysis. Immune cells such as lymphocytes were not separated from other MNC’s when assessing DNA methylation. This made it difficult to verify expression of candidate genes by cell type for gene expression analysis. Furthermore, since DNA methylation are cell and tissue-specific, future studies should ensure that DNA methylation analysis is performed in a specific cell type and avoid contamination with other cell types. Gene expression in cord MNCs were generally quite low, making detection difficult. Nevertheless, our ddPCR method allowed for more accurate measurement of low gene expression as well as low cDNA concentrations in comparison to other methods such as RT-qPCR. Hypo- and hypermethylated genes that were generated by a T-test did not show statistical significance after multiple comparison testing using the Benjamini-Hochberg correction; however, we mitigated this limitation by testing for changes in gene expression through ddPCR. Another limitation may be with the calculation of birth weight percentile. Despite having found borderline statistical significance, the calculation was performed manually using a growth chart and may confer a false positive finding. Using z-scores from the World Health Organization’s database may allow for more precise birthweight percentile measurement. This calculation is currently underway. Our sample size for gene-specific DNA methylation analysis was small primarily due to matching cases with controls, the availability of DNA sample and cost. The sample size for gene expression analysis was even smaller due to poor RNA quality obtained from cord MNCs. Finally, a larger sample size would have been needed to elucidate a clearer picture on the associations with B\textsubscript{12} and folate on DNA methylation and on gene expression. Nevertheless, our exploratory approach in this study may be used as a foundation for future studies in order to confirm our findings.
5.3 Future Directions

Our data provide a framework for future large-scale studies aimed at elucidating the relationship between maternal B₁₂ and folate status and DNA methylation in human offspring and their functional, biological and clinical ramifications and long-term health consequences. Some potential future studies include: 1) collection of a larger sample of Canadian pregnant women, with higher quality infant RNA from cord blood lymphocytes, and confirm DNA methylation status with bisulphite pyrosequencing of the fourteen genes identified as most relevant to infant growth and development, and gene expression with ddPCR; 2) design and implement follow up studies of the infants at early childhood to investigate the effect of B₁₂ and folate on DNA methylation, anthropometric and metabolic outcomes, and health and disease outcomes related to growth and development such as with insulin resistance and adiposity; and 3) design a similar study in an animal model to investigate more causal relationships between B₁₂ and folate status, DNA methylation and gene expression.
5.4 Final Conclusions

To conclude, this research project provides novel information regarding the effect of B₁₂ and folate status on DNA methylation and genes associated with infant growth and development. We measured genome-wide and gene-specific methylation in cord blood MNCs, as well as gene expression on selected genes with functional significance to metabolism and development. Overall, our findings support the importance of nutrient induced epigenetic changes that can affect infant health at birth, early childhood, and can potentially modulate chronic disease risk in adulthood (Figure 5.1).
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Appendix

Appendix A. Analysis of blood biomarkers

Aliquots of maternal and cord blood stored in biorepository were used to analyse blood biomarkers of the PREFORM study folate and B\(_{12}\) measurements.

<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(_{12})</td>
<td>Serum B(_{12})</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(_{6}))</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>Global DNA methylation and hydroxymethylation</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td></td>
<td>38 SNPS in 26 one-carbon nutrient related genes</td>
<td>Genotyping</td>
</tr>
</tbody>
</table>
Appendix B. Final propensity score model in SAS code

```sas
proc logistic descending data = cutoffs;
Model case = a_b6 infant_sex a_tchol mom_age caesarian preg_smoke
preg_smoke*caesarian*a_b6;
Output out = propensity2 p = p;
run;

Data propensity2;
set propensity2;
propensity_score = log(p/(1-p));
run;
proc means data = propensity2 std;
var propensity_score;
output out=temp std=std;
run;
data temp;
set temp;
std1=0.2*std;
run;
proc print data= temp;
run;

%gmatch (data = propensity2,
Group = case ,
Id = subject_id,
Mvars = propensity_score a_bmi ,
Wts = 1 1 ,
Dmaxk =0.39568 3 ,
ncontls = 1 ,
Seedca = 1234 ,
Seedco = 5678 ,
Print = y ,
Out = matches);
```
Appendix C. Histograms of CpG island and non-CpG island methylation

Illumina Infinium MethylationEPIC Array contains additional probes in non-CpG islands resulting in a bias towards hypermethylated sites. This was controlled for in the analyses by separating the global DNA methylation analysis by total, CpG island and non-CpG island sites. GenomeStudio was used to generate bar graphs for all 44 subjects showing the distribution of DNA methylation depending on CpG site probe location. The dataset with the total amount of CpG sites (Appendix Figure 1B) shows that in both cases and controls, there is a high occurrence of both hypo- and hypermethylation. The level of hypermethylation decreases when only accounting for CpG islands only (Appendix Figure 2B). In the non-CpG island dataset (Appendix Figure 3B), the additional probes are clearly depicted with a high level of hypermethylation like the total dataset.

Appendix Figure 1C. Total CpG site dataset including CpG and non-CpG islands. The x-axis indicates the level of DNA methylation and the y-axis indicates the number of occurrences. Cases are shown on the left and controls on the right.
Appendix Figure 2C. CpG island only dataset. The x-axis indicates the level of DNA methylation and the y-axis indicates the number of occurrences. Cases are shown on the top and controls on the bottom.

Appendix Figure 3C. Non-CpG island only dataset. The x-axis indicates the level of DNA methylation and the y-axis indicates the number of occurrences. Cases are shown on the top and controls on the bottom.
### Appendix D. IPA functional analysis summary table of all four datasets

<table>
<thead>
<tr>
<th>IPA Functions</th>
<th>Mann-Whitney test</th>
<th>T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Datasets</strong></td>
<td><strong>371 Total</strong></td>
<td><strong>219 Hypo only</strong></td>
</tr>
<tr>
<td><strong>Diseases and Disorders</strong></td>
<td>Cancer, GI, Endocrine, Organ injury and abnormalities</td>
<td>Cancer, GI, Endocrine, Organ injury and abnormalities</td>
</tr>
<tr>
<td><strong>Molecular and Cellular Functions</strong></td>
<td>Cellular movement, Cell cycle, growth and proliferation, function and metabolism</td>
<td>Cell movement and cycle, Lipid metabolism, Small molecule biochemistry</td>
</tr>
<tr>
<td><strong>Physiological System Development and Function</strong></td>
<td>Hematological, Reproductive, Cardiovascular, Tissue and Embryonic</td>
<td>Tissue, Organismal, Connective Tissue, Cardiovascular, Skeletal</td>
</tr>
<tr>
<td><strong>Networks</strong></td>
<td>Cancer, Connective Tissue disorders, Cell signalling, Developmental disorder</td>
<td>Cell death, Development, Growth, Proliferation, Movement, Cancer, Molecular Transport</td>
</tr>
</tbody>
</table>