THE EFFECT OF PATERNAL FOLIC ACID SUPPLEMENTATION ON FOLATE CONCENTRATIONS AND DNA METHYLATION OF THE OFFSPRING IN A MOUSE MODEL

by

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

Nutritional Sciences

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Abstract

**Background:** DNA methylation is programmed during embryogenesis, and folate is critical in the one-carbon transfer reactions. Maternal folic acid (FA) supplementation has been shown to significantly modify offspring DNA methylation. However, the role of paternal folate status is unknown. We investigated the effect of paternal FA supplementation on offspring DNA methylation.

**Methods:** Weanling male C57BL/6 mice (n=20) were randomized to 2 or 20 mg FA/kg diets. After 3-4 weeks, breeding began with females on the control diet. At birth, pups were sacrificed, and plasma and tissues collected. Folate concentrations were determined by standard microbiologic assay, and \( Ppar-\alpha, Ppar-\gamma, Gr \) and \( Igf-2 \) methylation by bisulfite conversion and pyrosequencing.

**Results:** Paternal supplementation significantly decreased the breeding period \( (p < 0.001) \). There was a significant sex effect on \( Igf-2 \) methylation, and supplementation significantly increased \( Gr \) methylation \( (p < 0.05) \).

**Conclusions:** Paternal FA supplementation may improve breeding and modulate methylation of essential genes involved in the offspring’s development.
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This thesis is dedicated to the joyful pursuit of discovering the truth in all things. 1 Cor 3:16
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Abbreviations

A<sup>vy/a</sup>: viable yellow agouti
BCRP: breast cancer resistance protein
BMI: body mass index
BORIS: brother of the regulator of the imprinted site
CHD: coronary heart disease
CHMS: Canadian Health Measures Survey
CpG: cytosine guanine dinucleotide
CRC: colorectal cancer
CTCF: CCCTC-binding factor
CVD: cardiovascular disease
DFE: dietary folate equivalent
DHF: dihydrofolate
DHFR: dihydrofolate reductase
DNMT: DNA methyltransferase
dTMP: deoxythymidine-5-monophosphate
dUMP: deoxyuridine-5-monophosphate
ESCC: esophageal squamous cell carcinoma
10-formylTHF: 10-formyltetrahydrofolate
FBP: folate binding protein
FD: folate-deficient
FPGS: folylpolyglutamyl synthase
FR: folate receptor
FS: folate-supplemented
GCPII: glutamate carboxypeptidase II
GGH: γ-glutamyl hydrolase
Hcy: homocysteine
HFD: high fat diet
HNSCC: head and neck squamous cell carcinoma
ICR: imprinting control region
JHMD2A: Jumonji C-terminal containing histone demethylase A
LINE-1: long interspersed nuclear element-1
LPD: low protein diet
MAT: methionine adenosyltransferase
MBD2: methyl-CpG binding domain protein 2
MD: maternal deficient
Met: methionine
MRP2: multidrug resistance-associated protein 2
MS: methionine synthase
MS: maternal supplemented
5-methylTHF: 5-methyltetrahydrofolate
MTHFR: methylenetetrahydrofolate reductase
MTRR: methionine synthase reductase
NA: not applicable
NSCLC: nonsmall cell lung cancer
NTD: neural tube defects
OATP1B1: organic anion-transporting polypeptides 1B1
OATP1B3: organic anion-transporting polypeptides 1B3
PABA: para-aminobenzoic acid
PCFT: proton coupled folate transporter
PD: paternal deficient
POMC: pro-opiomelanocortin
PS: paternal supplemented
RBC: red blood cell
RDA: recommended dietary allowance
RFC: reduced folate carrier
SAH: S-adenosylhomocysteine
SAM: S-adenosylmethionine
SHMT: serine hydroxymethyltransferase
TET: ten-eleven translocation
THF: tetrahydrofolate
TS: thymidylate synthase
UL: upper limit
UMFA: unmetabolized folic acid
Chapter 1: Introduction

Folate, a water-soluble vitamin B naturally found in foods, and folic acid (FA), the synthetic form of folate used in supplements and fortified foods, are important in human health and diseases including macrocytic anemia, cardiovascular disease, neural tube defects (NTDs) and congenital disorders, cognitive impairment, and cancer (1). Mandatory FA fortification was implemented in 1998 based on an overwhelming body of evidence that suggested a protective effect of periconceptional FA supplementation on NTD risk (2). FA fortification has achieved its primary objective with national surveys reporting a 15-50% reduction in the NTD rates in North America post-fortification (3). In addition, ~40% of the North American population are reported to use FA containing vitamin supplements for possible but as yet unproven health benefits (4). Consequently, for the past 18 years, the Canadian population has experienced a significant increase in intakes and blood levels of folate and FA. According to the Canadian Health Measures Survey data from 2007 to 2009, almost all Canadians have red blood cell (RBC) folate concentrations above the deficiency cut off of 305 nmol/L (5) and over 40% of the Canadian population have RBC folate concentrations above the level considered to be high (1360 nmol/L; the 97th percentile of the NHANES) (2,6). Although increased folate and FA intakes and blood levels may provide health benefits, an emerging body of evidence has suggested potentially serious adverse health effects of high intake and levels of FA, including cancer promotion and epigenetic alterations (7–9).

DNA methylation is an important epigenetic determinant in gene expression, DNA integrity and stability, and chromatin modifications (1). It is mechanistically related to the development of chronic diseases including cancer. DNA methylation is programmed during embryogenesis and establishes a new pattern that is maintained through early life and adulthood. It has been shown that maternal nutrition during pregnancy can modulate DNA methylation in the offspring (10–12), thereby influencing the risk of obesity, type 2 diabetes and cancer in adulthood.

Folate plays an important role in biological methylation reactions including DNA methylation (1). Folate, in the form of 5-methyltetrahydrofolate (5-methylTHF), contributes a methyl group to the remethylation of homocysteine to methionine, a precursor of S-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions. Folate deficiency and FA supplementation imposed postnatally or in adulthood have been shown to affect global and gene-specific DNA methylation, although the effects appear to
be gene and site-specific and depend on species, cell type, target organ, and stage of transformation as well as on the timing, degree and duration of folate intervention (1). Proof-of-principal studies have shown that maternal supplementation of methyl group donors (FA, vitamin B12, methionine, choline) can increase DNA methylation in the CpG promoter regions of the Agouti and AxinFused genes, resulting permanent phenotypic changes and disease susceptibility in rodents (13,14). Kim lab has previously shown maternal FA supplementation alone can also significantly modify global and gene-specific DNA methylation in the offspring (15) with changes in cancer susceptibility (10). Other groups have also demonstrated that maternal FA supplementation either alone or in conjunction with other dietary manipulations can change DNA methylation in the offspring (10). A growing body of studies investigating the effect of maternal FA supplementation on DNA methylation in the offspring is related to the concern that the developing fetus in North America has been exposed to high levels of folate and FA in utero due to FA fortification, prevalent FA supplement use, and public health guidelines recommending periconceptional FA supplementation to reduced NTD risk.

Studies have begun to unravel the role of paternal nutrition on epigenetic alterations in the offspring, including those of DNA methylation (16–18). However, the potential role of paternal folate status in DNA methylation in the offspring is largely unknown at present (7). Males are likely to be exposed to environmental factors leading to folate insufficiency, including smoking (19), alcohol consumption (20), and low intake of foods containing folate (8). Folate inadequacy in males may affect reproductive functions (21,22) including spermatogenesis and sperm quality, and it has been suggested that paternal folate status significantly affects folate metabolism in the placenta (9). A preliminary study reported paternal folate deficiency modulates global DNA methylation and IGF-2 expression in the rat fetal brain (10). However, it is unknown whether paternal FA supplementation during mating affects DNA methylation in the offspring.

Given this consideration, the main objective of this thesis was to investigate the effects of paternal FA supplementation on plasma and hepatic folate concentrations and gene-specific DNA methylation in the liver of the offspring in a mouse model.
Chapter 2: Literature review

2.1 Folate and folic acid

2.1.1 Overview

Folate, an essential water-soluble B vitamin, is naturally present in commonly consumed foods, and folic acid, the synthetic form of folate, is used in supplements and food fortification. The importance of folate and folic acid in human health is attributed to their role in one-carbon transfer reactions involved in biological methylation reactions and nucleotide biosynthesis. Thus, folate plays a significant role in DNA synthesis, stability and repair, and DNA methylation, aberrancies of which are mechanistically related to the development of human diseases. In fact, studies have suggested associations between folate deficiency and the development of many health conditions, including megaloblastic anemia, coronary heart disease and stroke, neural tube defects (NTDs) and other congenital disorders, adverse pregnancy outcomes, neuropsychiatric disorders, cognitive impairments and the risk of several cancers. However, the exact nature and magnitude of these purported associations between folate status and the development of these conditions and potential underpinning mechanisms have yet to be clearly elucidated.

With regards to the source of folates in the human diet, they are found in many foods, including green leafy vegetables, citrus fruits, organ meats and legumes. The highest concentrations of folate are in yeast, spinach, liver, peanuts, lima and kidney beans and broccoli. Due to the observed association between low maternal folate levels and the risk of NTDs, the Canadian government implemented fortification with folic acid of white wheat flour and other selected grains in 1998. Consequently, folate deficiency is virtually nonexistent in the Canadian population according to the results from the 2007-2009 Canadian Health Measures Survey (CHMS), and more importantly, the prevalence of NTDS was reduced by 46%. However, what seems to have been a great solution to a major concern for newborn infants is not without its own set of concerns. Both folic acid fortification and prevalent supplement use have raised the consumption of folate and folic acid, and this is reflected in the high blood concentrations of folate and the appearance of unmetabolized folic acid (UMFA) in the population. In the post-fortification era, concerns have been raised with regard to the potential adverse consequences of excessive intakes and blood levels of folate and folic acid.
2.1.2 Chemical structure

Folate is a generic term used to refer to a group of compounds with similar chemical structures and nutritional properties (1). Folates are consisted of three moieties: the pterin (also known as pteridine) ring that is linked via a methylene bridge to para-aminobenzoic acid (PABA), which is connected via a peptide bond to a glutamic residue (Figure 2.1).

![Figure 2.1: Chemical structure of folic acid [A] and folate [B].](image)

Three moieties are joined together in folate: in order, an aromatic pteridine ring, para-aminobenzoic acid (PABA) and glutamic acid. FA is fully oxidized and monoglutamylated. Naturally occurring folates are reduced and polyglutamylated (with up to nine glutamate residues). One-carbon units can be added to THF at N⁵ and N¹⁰: R = CH₃ (N⁵), CHO (N⁵ & N¹⁰), CH=NH (N⁵), CH₂ (N⁵ & N¹⁰) and CH= (N⁵ & N¹⁰). Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd): (32). Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Folic acid, the synthetic form, contrasts with other naturally occurring folates in the oxidation state of the pteridine ring, as folic acid is fully oxidized while folates are generally reduced (1). Furthermore, folic acid is monoglutamylated, whereas naturally occurring folates consist of a polyglutamylated chain with up to nine glutamate residues linked via γ-peptide bonds. Tetrahydrofolate (THF) has the capacity to add one-carbon units on to its N-5 and N-10 positions and transfer them for one-carbon metabolism involved in de novo nucleotide biosynthesis and biological methylation reactions (1) (Figure 2.2).
Figure 2.2: Folate absorption, transport and biochemical functions. Intracellular uptake and metabolism of folate, and its role in one-carbon transfer reactions. Glutamate carboxypeptidase II (GCPII) is involved in intraluminal folate hydrolysis; folate receptor (FR), reduced folate carrier (RFC) and proton coupled folate transporter (PCFT) facilitate intracellular folate uptake; folylpolyglutamyl synthase (FPGS) polyglutamylates monoglutamyl folates for intracellular retention and accumulation; \( \gamma \)-glutamyl hydrolase (GGH) catalyzes the hydrolysis of polyglutamates for cellular efflux. Methionine synthase (MS), methionine synthase reductase (MTRR) and methylenetetrahydrofolate reductase (MTHFR) are involved with the methionine cycle; dihydrofolate reductase (DHFR), serine hydroxymethyltransferase (SHMT) maintain the intracellular folate pool. Thymidylate synthase (TS) facilitates nucleotide biosynthesis, DNA methyltransferases (DNMT1, 3a, 3b) mediate DNA methylation; methyl-CpG binding domain protein 2 (MBD2) and DNA demethylase reverse methylation. Deoxythymidine-5-monophosphate (thymidylate; dTMP); deoxyuridine-5-monophosphate (dUMP); homocysteine (Hcyt); methionine (Met). A filled circle represents a pteridine ring bound to para-aminobenzoic acid. A filled triangle represents a glutamate, linked by peptide bonds to form different chain lengths of polyglutamylated folate. Adapted and reprinted by permission from the publisher (John Wiley and Sons Ltd): (32). Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
2.1.3 Absorption, metabolism and biochemical functions

2.1.3.1 Folate absorption

Intake of folate from dietary sources and folic acid from fortified food or supplements is essential since mammals are unable to synthesize folates (32). Folate absorption occurs mostly in the proximal small intestine, in the duodenum and proximal jejunum (33). Although there are many facilitative solute carriers for folate in the proximal region of the small intestine, protein coupled folate transporters (PCFT or SLC46A1) and reduced folate carriers (RFC or SLC19A1) are specific to folate (33,34). Due to its wide expression in the apical brush border membrane of the intestinal mucosa, RFC was widely accepted to be the primary transport system of folate absorption in the small intestine. However, PCFT was subsequently identified as the primary transport system for folate absorption in the small intestine (35). Folate transport by both transporters is highly pH-dependent, and the distinguishing factor between them is that RFC has a pH optimum of 7.4 while PCFT has a pH optimum of 5.5, making it more efficient in the acidic environment of the small intestine (36).

RFC is a facilitative carrier with 12 transmembrane domains, with the N and C termini directed towards the cytoplasm (37). It has a high affinity for reduced folates, 5-methylTHF and 5-formylTHF. The gene, SLC19A1, encoding RFC is located on chromosome 21q22 in humans (37). RFC is expressed in almost all tissues and cell lines, thus it plays a significant role in folate delivery to cells in the systemic circulation (38). RFC is highly expressed in several epithelial cells, including the apical brush-border membrane of gastrointestinal cells, basolateral membrane of proximal renal tubule, apical membrane of choroid plexus and retinal pigment epithelium (38). RFC is an organic phosphate antiporter that transports substrates like pyrophosphate derivatives out of the cell while using the energy from the downhill flow of organic phosphates to transport folates into cells (37,39).

The gene, SLC46A1, encoding PCFT is located on chromosome 17q11.2 in humans, and, like RFC, has 12 transmembrane domains with N, C termini in the cytoplasm (40–42). This transporter is expressed largely in the duodenum and jejunum, the liver and at much lower levels in the colon (33,40). PCFT has a high affinity for folic acid, and its maximum activity is at a pH of about 5.5 for most cells (36,43). The level of activity increases with a decrease in pH since folate transport by PCFT is electrogenic and protein coupled, whereby the transport is accompanied by acidification (33).
Both RFC and PCFT are highly specific for the monoglutamate form of its substrates. Since dietary folates are largely in their polyglutamate form, in the small intestine, polyglutamylate chain of folates are first hydrolyzed to folates with no more than three glutamate residues by glutamate carboxypeptidase II (GCPII), an enzyme located on the brush border membrane of intestinal cells with an optimal activity at pH of 6.5 (Figure 2.2) (38,44). Hydrolysis of the polyglutamate involves cleavage of the terminal glutamate moiety to yield a monoglutamate (45).

As previously mentioned, the main transporter of reduced folates across the intestinal apical cellular membrane is the proton-coupled folate transporter (PCFT). However, other transporters including RFC and the folate receptors (FR-α, β, γ) are also involved, albeit to a lesser degree. FRs exhibits high affinity for all forms of folate, although its highest affinity is for folic acid (35).

In the small intestine, following intracellular uptake of the monoglutamates, they are quickly polyglutamylated for intracellular retention via folylpolyglutamyl synthase (FPGS) by adding 4-8 glutamate residues to form long side chains (Figure 2.2) (31). These polyglutamates are better substrates than monoglutamates for intracellular folate-dependent enzymes involved in one-carbon transfer reactions (46). On the other hand, for the purpose of efflux from the cell via RFC, γ-glutamyl hydrolase (GGH) removes the terminal glutamates from the polyglutamylated forms of folate (35). The predominant form of circulating folate is 5-methylTHF, of which about a third generally remains unbound, half are bound to albumin, and only a small fraction is attached to specific folate-binding proteins, mainly FR-γ (35).

In other tissues such as the kidneys and the liver, which store high levels of polyglutamylated forms of folate, GGH hydrolyzes these folates into monoglutamylated forms that are transported out of the cells into to meet systemic folate demands (47,48). In the liver, once polyglutamylated folates are hydrolyzed into monoglutamates, they are either secreted in the bile into the small intestine for reabsorption and thereafter into the portal and hepatic veins, or transported across the basolateral membrane into hepatic sinusoids under conditions of folate deficiency (38). Although PCFT and RFC are expressed in the liver, their role in the folate transport remains unclear (33,40). The mediators involved in hepatic folate uptake are multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP), which are expressed in the apical bile canalicular membrane (49,50). It has been established that MRP2
mediates 5-methylTHF uptake into bile canalicular membrane vesicles (51). On the other hand, the mediators involved in hepatic folate export are organic anion-transporting polypeptides 1B1 (OATP1B1) and 1B3 (OATP1B3), which are expressed on the basolateral membrane of the hepatocyte alongside hepatic sinusoids (52–54). Following uptake of a variety of substrates from sinusoidal blood into the hepatocytes, OATP1B1 and OATP1B3 are liver-specific anion transporters that export folates across the bile canalicular membrane (55–57). However, the form of folate that is transported has yet to be determined. All four mediators work together for the process of folate uptake from hepatic venous blood into hepatocytes and export into bile, followed by delivery to the intestine where folates are either absorbed or excreted (38).

Although PCFT expression in the colon is very low, folate transport activity at a pH of 5.5 in the apical brush-border membrane of proximal and distal colon has been observed in humans (58). The role of the colon in folate transport is an emerging area of interest as recent studies in humans have demonstrated that a significant portion of folate is absorbed in the colon (59,60). The colon is a potentially important endogenous source of folates because of its rich population of bacteria that are capable of synthesizing folate de novo (61). Bacterially synthesized folates have previously been shown be absorbed in the colon and incorporated in the host tissues (62).

2.1.3.2 Folate metabolism

For its role in biochemical reactions, folic acid is first reduced dihydrofolate (DHF), then to tetrahydrofolate (THF) by DHF reductase (DHFR), and methylated to the metabolically active 5-methylTHF (Figure 2.2) (1). The aforementioned modifications occur primarily in the small intestine and to a lesser degree, in the liver (63).

One-carbon transfer reactions are dependent on folate, which covalently binds to one-carbon units at N⁵ and N¹⁰ (Figure 2.1). Folate participates in critical one-carbon metabolisms, including the methionine cycle, biological methylation reactions and nucleotide biosynthesis (64). In the methionine cycle, the only reaction that produces 5-methylTHF is the reduction of 5,10-methyleneTHF via methylenetetrahydrofolate reductase (MTHFR) (Figure 2.2). In vivo, this reaction is irreversible (65). Methionine synthase reductase (MTRR) then activates methionine synthase (MS), an enzyme dependent on the co-factor cobalamin (vitamin B₁₂), which in turn converts homocysteine (Hcy) to methionine. The enzyme methionine adenosyltransferase (MAT) and ATP can then activate the de novo synthesized methionine to

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generate S-adenosylmethionine (SAM), the universal methyl donor for most biological methylation reactions (65).

DNA methylation occurs on the cytosines within cytosine guanine dinucleotide (CpG) sequences and is mediated by a group of enzymes called DNA methyltransferases (DNMTs) (Figure 2.2) (1). Once DNMTs use SAM as a methyl donor, SAM is converted to S-adenosylhomocysteine (SAH), which is hydrolyzed back to Hcy to begin a new remethylation cycle (46). DNMT3a and 3b play a role in de novo methylation and DNMT1 is responsible for the maintenance of CpG methylation (1). Demethylation is mediated by both passive and active processes including one mediated by a purported active DNA demethylase, methyl-CpG binding domain protein 2 (MBD2) (66). Recently, ten-eleven translocation (TET) proteins have been identified to have a key involvement in cytosine demethylation as they are dioxygenases that catalyze the oxidation of 5-methylcytosines to 5-hydroxymethylcytosine (67,68). Mutations of TET genes have been observed in the development of human cancers (69).

Serine hydroxylmethyltransferase (SHMT), which is dependent on vitamin B₆ as a cofactor, catalyzes the reversible interconversion of serine and glycine (Figure 2.2) (46). In pyrimidine biosynthesis, thymidylate synthase (TS) mediates the transfer of a methyl group from 5,10-methyleneTHF to deoxyuridine monophosphate (dUMP). This one-carbon transfer reaction generates DHF and deoxythymidine monophosphate (dTMP, thymidylate) a precursor of pyrimidylate biosynthesis (46). DHFR catalyzes the reduction of DHF to THF, and MTHFR can irreversibly convert 5,10-methyleneTHF to 5-methylTHF. Additionally, 10-formyltetrahydrofolate (10-formylTHF) is an important substrate of essential enzymes in purine biosynthesis, including phosphoribosylaminomimidazolecarboxamide formyltransferase and methionyl-tRNA formyltransferase (1).

2.1.4 Dietary sources and requirements

The human gastrointestinal tract is colonized by an array of gut microbiota that are capable of producing nutrients including various vitamins (70). Many species within the genera *Bifidobacterium* and *Lactobacillus* have been established to be folate producers via condensation of PABA with 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (70,71). Because humans themselves cannot synthesize folate, they maintain adequate status through dietary or supplemental sources of the vitamin (72). Foods containing folate include, and are not restricted to, lentils and romano beans, black and white beans, okra, asparagus, spinach, salad
greens, pasta made with enriched wheat flour, avocado, sunflower seeds, Brussels sprouts, beets, broccoli, eggs, corn and fruits (73). Due to the low stability of naturally occurring folates and a significant proportion of folate lost via food harvesting, storage, processing and preparation, it is generally estimated that the bioavailability of food folate is 25%-50% that of folic acid (27,74). Dietary recommendations are established based on the amount of dietary folate equivalents (DFEs) required to maintain normal red blood cell concentrations (range, 160-600 µg/L) (27). They are expressed as DFEs in order to account for the difference in bioavailability of food folate and folic acid (73).

1 µg of DFE = 1 µg of food folate

1 µg of DFE = 0.6 µg of folic acid from fortified food or a supplement taken with meals

1 µg of DFE = 0.5 µg of folic acid supplement taken on an empty stomach

The Recommended Dietary Allowance (RDA) for both men and women is 400 µg DFEs/day. For pregnant women and women during lactation, the RDA is increased to 600 µg DFEs/day and 500 µg DFEs/day, respectively (27). The tolerable upper intake level (UL; upper limit) for folic acid, which was set to minimize masking of vitamin B12 deficiency, is 1 mg folic acid/day for both adult men and women (27). However, the UL for dietary folate has not been established (27).

2.1.5 Measurement of folate status

There are typically two measures of folate status, red blood cell (RBC) and serum or plasma folate concentrations (75). Due to intracellular folate uptake of RBCs that only occurs during erythropoiesis and the low expression of membrane-associated FRs of mature RBCs, these mature RBCs do not take up a significant amount of folate (76). In fact, the half-life of RBCs is 60 days (77), and kinetic studies have demonstrated that, following a change in dietary folate intake, it would take four half-lives (about 35 weeks) for RBC folate to reach steady-state levels (78). Therefore, RBC folate concentrations change relatively slowly after dietary intake (75) and indicate long-term folate status (79) or storage.

On the other hand, serum (or plasma) folate is considered to be an indicator of recent folate intake (27). Serum levels peak 1-2 hours following folate ingestion (80), and the half-life of monoglutamylated folates is a few hours long (81). Although a single measurement of serum
folate is insufficient to reflect a chronic deficient state, multiple measurements over the course of one month can indicate low folate status or folate deficiency (82). According to the World Health Organization (WHO), the cut-off values of serum and RBC folate concentrations for folate deficiency are <10 nmol/L and <340 nmol/L, respectively (79).

In addition to the two measures of folate status described above, plasma Hcy concentration is an inverse indicator of folate status. With low folate levels, there is a decrease in the supply of 5-methylTHF, a cofactor in the conversion of Hcy to methionine (83). Consequently, Hcy concentration rises, and the rise in circulating plasma Hcy levels (>14 nmol/L) is a functional biomarker of folate deficiency (84). It is, however, important to note that the increase in Hcy levels is not restricted to the indication of folate deficiency since low intake of other vitamins, such as B₆ and B₁₂, is also associated with elevated Hcy concentrations (27).

Although there is no established upper cut-offs for RBC and serum folate concentrations that are associated with adverse health effects, some arbitrary cut-offs used in scientific literature are RBC folate concentrations >1360 nmol/L (reflecting the 97th percentile from the 1999-2004 National Health and Nutrition Examination Survey [NHANES]) and serum folate concentrations > 45 nmol/L (the highest calibration point in the assays for serum folate measurement in NHANES 1991-1994 and 1999-2000) (2). Although high intake and blood levels of folate have been linked to adverse health outcomes, such as cognitive decline, masking of vitamin B₁₂ deficiency, and time- and dose-dependent acceleration of tumor progression (31,85), the health concerns specific to an individual’s blood folate levels above these cut-off values have yet to be established.

2.1.6 Intake and blood levels of folate and folic acid in the North American population

In North America, food fortification with folate was implemented in 1998 based on an overwhelming body of evidence that indicate that periconceptional folic acid supplementation can reduce the risk of neural tube defects (NTDs) (27,86,87). The relationship between low folate status and adverse pregnancy outcomes started to appear in literature in the 1960’s (88–90). Because of the important role of folate in nucleotide biosynthesis, folate deficiency can impair cell growth and replication and adversely affects fetal and placental development (91). Of the adverse birth outcomes associated with folate deficiency, the most concerning was NTDs, characterized by a failure of early closure of the neural tube in embryonic development. As a result, there is damage to the exposed underlying neural tissue, and the most severe case is
anencephaly, which leads to death (92). Other lesions, such as spina bifida, are associated with a range of morbidities depending on the location and severity, including urinary and fecal incontinence and limb paralysis (92). A number of placebo-controlled randomized clinical trials have unequivocally established the protective effect of periconceptional folic acid supplementation on NTDs (93,94). Additionally, Daly and colleagues have established the dose response relationship between maternal RBC folate levels and NTD risk, where 900 nmol/L is accepted as optimal for the minimization of NTD risk in the offspring (95). More recently, Hopkins and colleagues determined the predictability of RBC folate concentration distributions alone for NTD prevalence and its change associated with folic acid fortification. They concluded that RBC concentrations above 1000 nmol/L are optimal for reducing the risk of NTDs associated with maternal folate deficiency (96).

Although the initial government policy to prevent NTDs was to make a recommendation for increased folate intake to women of childbearing age, these educational measures proved to not be as effective as intended. In 1991, the Center for Disease Control and Prevention recommended that women with previous NTD-affected pregnancies consume 400 µg of folic acid/day from the day of deciding to plan a pregnancy (97). Subsequently in 1992, the United States (US) Public Health Service provided guidelines for all women of childbearing age to consume 400 µg of folic acid/day through supplements and diet (98), which were reiterated in 2009 by the US Preventive Services Task Force (99). However, these measures had several limitations. National guidelines did not effectively reach every high-risk population and individuals who experienced unplanned pregnancies (100,101). Moreover, studies showed that women planning a pregnancy tended not to make significant behavioural changes (102). In fact, no significant changes in NTD trends were observed after the recommendations regarding periconceptional folate and folic acid consumption were issues and even after several educational campaigns were conducted to implement these recommendations (103).

For the purpose of effectively reducing the incidence of NTDs, over 50 countries have regulations in place for mandatory fortification of wheat flour with folic acid (97). In 2006, the WHO and the Food and Agricultural Organization of the United Nations (UN) provided guidelines for countries to set the amounts of folic acid to be fortified in flour (104). In the US and Canada, the respective fortification levels are 140 µg and 150 µg folic acid per 100 g of white wheat flour and other selected grains (97). For women, those of childbearing age are suggested to consume foods rich in folate and a multivitamin supplement containing 400 µg
folic acid/day (105). For those at a greater risk of having a baby with a NTD, they are recommended to take supplements containing a higher dose of 4-5 mg folic acid/day (73).

According to the NHANES data from 2003-2006, about 35% of the U.S. population reported the use of dietary supplements containing folic acid (106). Of the 1296 pregnant women in NHANES 1999-2006, 78% reported supplement use in the previous month (107). The data also revealed that pregnant women who were supplement users differed from those who were not by nearly every characteristic. After multivariate adjustment except for income, those that reported supplement use were more likely to have at least some college education and to be in their third trimester (107). In this analysis, the majority of reported folic acid supplements for pregnant users contained \( \geq 800 \mu g \) folic acid (107) and most prenatal vitamin supplement in Canada contain 1000 \( \mu g \) folic acid. These facts indicate that most pregnant women or women of reproductive age in North America are taking much higher doses of folic acid supplementation than is currently recommended (i.e., 400 \( \mu g \)) (107).

The data from the 2007 to 2009 CHMS revealed that one in four Canadians aged 6 to 79 reported supplement use (108). A number of factors were associated with folic acid-containing supplement use; those in higher income quartiles, females, those with frequent fruit and vegetable intake, and those who reported active leisure time were significantly more likely to have taken a folic acid-containing supplement (108). Since national surveys generally do not evaluate micronutrient intake of pregnant women due to the small sample size, Masih and colleagues collected data on one-carbon nutrient intake of Canadian pregnant women (109). In the report, 60% of the 305 participants used a vitamin B-containing supplement at least once a week 30 days before pregnancy (109). The proportion of supplement users increased to 93% and 89% in early (0-16 gestational weeks) and late pregnancy (23-37 gestational weeks), respectively (109). Although the RDA of folate during pregnancy is set at 600 \( \mu g \) DFEs/day (15), because the dosage of prenatal vitamins is generally 1 mg folic acid, this study revealed that 85% of the women consumed folic acid solely from supplements at or above the UL (1 mg/day) (109). With the addition of dietary folate intake, folate and folic acid intakes among pregnant women and women of reproductive age in North America is likely high. The high FA consumption is of concern as this may be associated with adverse health outcomes to the pregnant woman and fetus and as such, there is a need to determine optimal dosages of folic acid in supplements and prenatal vitamins in pregnancy.
According to the NHANES data from 1988-2010, which reveals the impact of food fortification and supplement use, the serum and RBC folate concentrations post-folic acid fortification increased compared with those pre-fortification era (110). In every age group, following folic acid fortification in 1998, serum and RBC folate concentrations increased by more than double and 1.5x, respectively (2). From the CHMS data from 2007-2009, folate deficiency, defined as RBC folate concentration < 305 nmol/L (27), was virtually nonexistent in Canada post-folic acid fortification (5). In addition, the RBC folate concentrations of more than 40% of the population were > 1360 nmol/L, the high cut-off established from the 97th percentile of the NHANES data from 1999-2004 (5). Nevertheless, the RBC folate concentrations of 22% of the women of childbearing age were still < 906 nmol/L, one of the suggested RBC folate concentrations for minimizing the risk of NTDs (5).

A recent Canadian prospective study assessed dietary and supplemental intake of folate and folic acid of 368 demographically diverse pregnant women from Toronto, Ontario and measured maternal and cord blood concentrations of folate and unmetabolized folic acid (111). Plumptre et al. found that over 90% of the women took periconceptional folic acid-containing supplements, and the most common dosage was 1 mg/day. No subject was folate deficient, and all of the women had RBC folate concentrations above 1000 nmol/L, which is one of the previously mentioned cut-offs, below which the risk of NTDs in the newborn increases (96). Serum and RBC folate concentrations were measured at early pregnancy (12-16 weeks of gestation) and at delivery (38-42 weeks of gestation; late pregnancy). The geometric mean (95% CI) serum folate concentrations at early and late pregnancy were 51 nmol/L (49, 54 nmol/L) and 39 nmol/L (37, 41 nmol/L) respectively (111). The geometric mean (95% CI) RBC folate concentrations at early and late pregnancy were 2417 nmol/L (2362, 2472 nmol/L) and 2793 nmol/L (2721, 2867 nmol/L) respectively. Cord serum and RBC folate concentrations were measured for the newborn infant, and they were 64 nmol/L (61, 68 nmol/L) and 2689 nmol/L (2614, 2765 nmol/L) respectively (111). During pregnancy, maternal serum folate concentrations significantly decreased ($P < 0.0001$), while RBC folate concentrations significantly increased ($P < 0.0001$) (111). With respect to folic acid-containing supplement use, serum and RBC folate concentrations were significantly greater for users than non-users in late pregnancy ($P < 0.0001$), but there was no significant difference observed in early pregnancy. Additionally, in late pregnancy, measurements taken from the cord blood from mothers who used supplements were significantly greater serum ($P < 0.0001$) and RBC folate ($P = 0.0002$)
concentrations (111). When DHFR is saturated, unmetabolized folic acid (UMFA) in the plasma is detectable in maternal and umbilical cord blood samples. The UMFA concentrations in these samples in this study was greater than those previously reported in countries with lower folic acid supplement use or without folic acid fortification. However, the lower proportion of plasma UMFA in total cord blood folate suggests that the fetal storage of folate is limited, and UMFA is more efficiently metabolized in the fetus for immediate use than in an adult (111).

The RBC folate concentrations of supplement users and nonusers in this study are greater than those reported using the NHANES data from 1999 to 2006 that showed the median (95% CI) RBC folate concentrations to be 1628 nmol/L (1589, 1695 nmol/L) for supplement users and 1041 nmol/L (962, 1184 nmol/L) for nonusers (107). The difference may be attributed to the greater proportion of women in the current study using supplements during the first trimester (93% vs. 55%) and the higher dose of folic acid in the supplements (1000 µg vs. 800 µg) (111). For similar reasons, maternal and cord RBC folate concentrations were higher in this study than previously reported in other countries with mandatory or voluntary fortification (112–114). Consistent with previous studies, serum folate was significantly higher in cord blood than maternal blood at delivery. Therefore, the data from this study indicate that maternal RBC folate concentrations in a cohort of representative pregnant women in Canada with mandatory folic acid fortification and widespread prenatal vitamin use were in the high range, and maternal and cord RBC folate concentrations were higher than reported values from unfortified countries (111).

2.1.7 Folate and folic acid in health and disease

Both folate deficiency and supplementation can impact health and the development of diseases in humans. The role of folate deficiency has been well established for the development of macrocytic anemia and NTDs (27). In addition, folate deficiency has been linked to cardiovascular disease (CVD), congenital disorders, adverse pregnancy outcomes, neuropsychiatric disorders and cognitive impairments, although the purported association has not been consistent (27). Epidemiologic and experimental studies have also shown a possible inverse association between folate status, as measured by dietary intake and blood levels, and the risk of several cancers (1).
2.1.7.1 Folate and cancer

Because of the important role of folate in nucleotide biosynthesis and biological methylation reactions of DNA, RNA, proteins and phospholipids, aberrancies of which contribute to the development of cancer, a potential role of folate status in cancer risk modulation has garnered a great deal of interest. In fact, a complex but possible inverse association between folate intake or blood levels and the risk of cancer has been suggested by a large body of epidemiologic and animal studies. While some studies have demonstrated an association between low folate status and cancers of the cervix, colorectum, lung, esophagus, brain, pancreas, prostate and breast (115), others have shown null associations (116,117). On the other hand, a number of studies also suggest the protective role of high folate status on cancer risk (118).

The influence of folate on cancer is complex because it varies depending on the cancer type. Kennedy and colleagues conducted a systematic review and meta-analysis to examine the relationship between folate intake levels and colorectal cancer (CRC) incidence (119). From the 27 studies included, the summary risk estimate for case control studies comparing high versus low total folate intake was 0.85 (95% CI: 0.81-1.05), suggesting that increased folate intake is protective against CRC development (119). The limitation of this review was that there was variability in the upper limit of FA intake in the studies. Wang and colleagues performed a meta-analysis of 10 prospective studies with data on 202,517 individuals in order to explore the association between dietary folate intake and prostate cancer risk (120). They determined that high dietary folate intake had little to no effect on the risk of prostate cancer (RR: 1.02; 95% CI: 0.95-1.09; p=0.598), and also does not have a significant effect on prostate cancer risk (RR: 1.01; 95% CI: 0.99-1.02; p=0.433). However, they found a statistically significant association between high serum folate levels and an increased risk of prostate cancer (RR: 1.21; 95% CI: 1.05-1.39; p=0.008) (120). They additionally indicated that a 5 nmol/L increase of serum folate levels was associated with an increased risk of prostate cancer (RR: 1.04; 95% CI: 1.00-1.07; p=0.0042). Therefore, this group showed that the role of folate in the risk of cancer is also dependent on the source of folate.

Two mechanisms have been proposed for the relationship between folate deficiency and the risk of cancer in normal tissues. With folate deficiency, when not all potential cellular folate-binding sites are saturated, folate-dependent pathways compete for the limited amount of 5-
methylTHF (121). With decreased amount of 5-methylTHF, there is a decrease in SAM, the universal methyl donor for most biological methylation reactions including DNA methylation. Lower levels of SAM should lead to DNA hypomethylation. Indeed, studies that utilized cell culture, animal and human models have shown that Hcy remethylation decreases with marginal folate deficiency (122). As a result, Hcy accumulates, and serum and cellular SAH levels rise because the equilibrium for the hydrolysis reaction favors SAH synthesis (123). Increased SAH levels impair essential methylation reactions of DNA and proteins through depressed SAM synthesis and inhibition of DNA methyltransferase (123). However, the effect of folate deficiency on tissue SAM and global DNA methylation has not been consistent. Genome-wide DNA hypomethylation is a consistent, early event in the development of most cancers (124). It is proposed that DNA hypomethylation is associated with genomic instability through changes in chromatin structure and increased mutation rates, both of which participate in the initiation and progression of cancer (125). DNA hypomethylation can also lead to inappropriate activation of certain proto-oncogenes, resulting in their expression and malignant cell transformation (126–128).

Another plausible mechanism by which folate deficiency may increase cancer risk in normal tissue is through impaired DNA synthesis and repair, resulting in DNA strand breaks and damage. As previously mentioned, folate is important for purine and pyrimidine nucleoside thymidine synthesis (129). The conversion of dUMP to dTMP via TS utilizes 5,10-methyleneTHF as a methyl donor. With folate deficiency, dTMP synthesis is limited and there is an accumulation of dUMP, which results in the incorporation of uracil into DNA. The normal repair mechanism for such instance is for DNA glycosylase to remove the misincorporated uracil (129). However, persistent folate deficiency and repeated patterns of uracil misincorporation into DNA and repair can lead to chromosomal damage (130–132). DNA instability increases the risk of transformation into malignancy (83). In vitro, animal and human studies (133–136) have demonstrated the significant association between low folate status and increased occurrences of DNA strand breaks, excessive uracil misincorporation into DNA, impaired DNA repair, and increased mutations, contributing to the development and progression of cancer.

On the other hand, folic acid supplementation has been shown to exert protective effects on cancer risk in normal tissues. One potential mechanism for this protective effect involves the maintenance of DNA fidelity, integrity and repair (137). Due to the important role of 5,10-
methyleneTHF in providing methyl donors for nucleotide synthesis, folic acid supplementation maintains the production of nucleotide precursors for DNA synthesis and replication. More specifically, folic acid supplementation increases intracellular 5,10-methyleneTHF concentrations, which may prevent the imbalance of nucleotide pools, specifically of uracil and thymidine, during DNA synthesis (138).

The effects of folate deficiency and supplementation on transformed or pre-neoplastic cells are opposite. While folate deficiency inhibits the progression of transformed cells, folic acid supplementation promotes the progression of transformed, (pre)neoplastic cells. The biologically plausible mechanism behind the inhibitory role of folate deficiency in transformed cells is that low levels of folate can reduce the supply of nucleotides for rapidly proliferating and transforming cells, causing inhibition of cell growth and replication. On the other hand, folic acid supplementation provides nucleotide precursors for the replication of transformed cells, causing even more rapid proliferation of neoplasms (138). Another plausible mechanism behind tumor inhibitory and promoting effects associated with folate deficiency and folic acid supplementation, respectively, is related to DNA methylation (138). In cancer cells, promoter CpG islands of several tumor suppressor and mismatch repairs genes are methylated, resulting in inactivation of these genes. Folate deficiency may lead to demethylation of these hypermethylated promoter regions of tumor suppressor and mismatch repair genes, thereby reactivating their function. On the other hand, folic acid supplementation may lead to de novo methylation of the promoter CpG island of tumor suppressor and mismatch repair genes, leading their inactivation. Although plausible, these mechanisms are yet to be demonstrated.

Several animal and epidemiologic studies as well as clinical trials have supported potential dual effects of folate status on cancer development and progression, depending on the dose and stage of cell transformation at the time of folate intervention. Chen et al. conducted a systematic review and meta-analysis of 16 prospective studies with a total of 744 068 participants to determine the association between folate intake and risk of breast cancer (7). They observed a U-shaped relationship between dietary folate intake and breast cancer risk. Women with daily dietary folate intake between 153 and 400 µg had a significantly lower risk of breast cancer compared to those women with daily dietary folate intake < 153 µg or > 400 µg. Zhang et al. conducted a meta-analysis of 9 prospective studies involving 570 000 individuals and examined the effect of folate intake on the incidence of lung cancer. They noted that high dietary folate intake had no significant effect on lung cancer risk, and additionally that a 100
μg/day increase in folate intake had no significant effect on the cancer risk. However, their further subgroup analyses indicated sex-specific effects of folate, where there was a protective effect of low folate intake (100-299 μg/day) in women and a protective effect of increasing daily folate intake by 100 μg in men (139).

2.1.7.2 Folate and cardiovascular disease

The interest in the relationship between folate and CVD followed the observation of a higher risk of vascular disease in individuals with untreated homocystinuria, a rare autosomal recessive condition where those affected have levels of plasma Hcy above 100 μmol/L (140). In 1976, Wilcken et al. first identified the association of circulating Hcy levels and chronic heart disease (141), and a number of subsequent studies indicated the lowering effect of reduced Hcy levels on the risk of chronic heart disease (142–144). In fact, the first meta-analysis of such retrospective studies, Boushey et al. noted a significant risk reduction of as much as 60% from every 5 μmol/L decrease in circulating Hcy levels (145). However, a more recent meta-analysis conducted by the Homocysteine Studies Collaboration in 2002 indicated a much weaker protective effect of reducing Hcy levels on cardiovascular, where every 3 μmol/L decrease in circulating Hcy levels, was associated with 10% reduced coronary heart disease (CHD) risk (144).

Folate concentrations are inversely related to circulating Hcy levels, and supplementation of B-vitamins including folic acid, vitamin B16 and B12 have been shown to lower Hcy levels (146). As a result, several randomized clinical trials were undertaken to determine whether folic acid supplementation, either alone or in combination with other B-vitamins, could reduce the risk of CHD and stroke. While the effects of supplementation on the primary prevention of CVD is currently unknown, studies have explored the effects of folic acid supplementation on the secondary prevention of CVD (147). In contrast to the overwhelming protective effect observed in case-control and prospective epidemiologic studies, randomized clinical trials of folic acid supplementation have generally shown a null effect of folic acid and other B vitamin supplementation on CHD (31,148). A meta-analysis of eight Hcy-lowering trials with a total of 37, 485 participants at increased risk CVD in 2010 assessed the effect of folic-acid-based B-vitamin in the prevention of cardiovascular events (first occurrence of non-fatal myocardial infarction or coronary death), stroke, cancer and all-cause mortality (149). This study showed that there was no significant effect of treatment with vitamin B on the risk of
CHD events (149). In 2012, Yang et al. conducted a meta-analysis of 26 randomized clinical trials, where they assessed the effects of treatment with folic acid supplementation (with or without additional B vitamin supplementation) for > 6 months on the risk of CVD, CHD or stroke among individuals at high risk of developing CVD (150). The results showed that folic acid supplementation was not associated with a significant reduction in the risk of CVD, CHD and all-cause mortality. Therefore, collective evidence suggests a null effect of folic acid supplementation on the secondary prevention of CHD.

Interestingly, Yang et al. demonstrated the protective effect of folic acid supplementation on the risk of future stroke risk, with a relative risk ratio (RR) (95% CI) of 0.93 (0.86-1.00; \( p =0.05 \)) (150). The observed disparity in the Hcy-lowering treatment with folic acid for the different vascular events is consistent with the previously mentioned report from the Homocysteine Studies Collaboration (144). The report suggested a stronger inverse relationship between Hcy levels and stroke than the one between Hcy levels and CHD, where there was a 19% decreased risk of stroke, compared to 11% decreased risk of coronary heart disease, for every 25% reduction in Hcy concentration (144). However, the findings for the effect of folic acid supplementation on stroke risk reduction remain inconsistent. Recently, Zhou et al. commented on the limitations of the studies that reported the protective effects of folic acid supplementation on the risk of stroke (151). The limitations are attributed to the lack of total Hcy levels when assessing stroke risk because the very high stroke risk associated with hyperhomocysteinemia involves total Hcy levels in the hundreds of micromoles per liter (typical range in the general population being 8-15 µmol/L) (152). Without being informed of the total Hcy levels, the implications of the protective effects of folic acid supplementation on stroke risk are unclear.

The precise mechanisms by which folate deficiency may increase the risk of, and folic acid supplementation may protect against, CHD and stroke have not been clearly elucidated. However, a number of studies have observed the possible antioxidative potential for folate, preventing foam cells from forming oxidized plaques on the endothelial wall (153,154). Some have shown that 5-methylTHF can reduce superoxide generation in vitro and in vivo (155,156), and others have demonstrated the dose-dependent protective effect of folate against oxidative modifications of low-density lipoprotein (LDL) (157).
2.1.7.3 Folate and cognitive function

Folate deficiency has been implicated in the manifestation of cognitive impairment, dementia, depression, peripheral neuropathy and spinal cord degeneration (158). Although findings are inconsistent, studies have indicated that, in patients with megaloblastic anemia, folate deficiency is associated with neurological complications that include depression and reduced cognitive function (159). Observational studies with epileptic, psychiatric and elderly individuals have shown that low serum, RBC and cerebrospinal fluid folate concentrations are associated with such neurological disorders. Additionally, folate deficiency has been linked with reduced response to treatment for major depressive disorders, cognitive decline and neuropathological indicators for Alzheimer’s disease and dementia (159). In fact, in individuals predisposed to psychiatric disorders, dietary folate deficiency can eventually exacerbate the underlying disorder (158).

There are three proposed mechanisms of the association between folate deficiency and cognitive dysfunction and neuropsychiatric disorders. First, reduced availability of SAM for methylation in the brain may lead to decreased levels of methylation of myelin and membrane phospholipids important in myelination and proper signalling in the nervous system (159). Second, decreased SAM impairs the monoamine pathways, and this effect has been shown to play a role in the development of depression (159). Last, the vascular and neurotoxic effects of low folate, such as oxidative stress and impaired glutathione metabolism, may contribute to reduced cognitive function (158).

Several studies have shown the protective effects of folic acid supplementation in patients with low mood and reduced cognitive function (159,160). Concerns have been raised as folic acid supplementation in the presence of vitamin B12 deficiency may lead to a relapse or progression of underlying cognitive impairment (158). Recently, calls for reconsideration of the UL of folic acid (1 mg/d for adults) have been proposed based on evidence of adverse neurological consequences from long-term use of folic acid supplementation between 0.5 and 1 mg in the presence of vitamin B12 deficiency (161).

2.1.7.4 Potential adverse effects of folic acid supplementation and/or high folate status

Although FA supplementation may provide some health benefits, there has been a rising concern for overconsumption of folate, in particular folic acid, from the use of supplements and food fortification. Although the accepted safe UL for FA intake is considered to be 1 mg/day for
adults (27), there is no set safe UL for naturally occurring folates (31). There is evidence in the literature that points to the potential harmful effects of FA supplementation, including masking of vitamin B12 deficiency and consequent irreversible neurological conditions (27); resistance or tolerance to antifolate drugs (162), impaired immune function (163), genetic selection of disease alleles (31), greater risk of diabetes (164) and asthma (165) for children born to mothers with high folate levels; cognitive impairments for the elderly who also have low vitamin B12 status (166); and tumor promoting effects on (pre)neoplastic lesions (1).

Integral to nonspecific immune response are natural killer (NK) cells that target and kill aberrant cells, such as tumorigenic and virally infected cells (167). Troen and colleagues examined the effects of folate status on NK cell cytotoxicity in postmenopausal women in the US following folic acid fortification (168). They reported an inverse U-shaped relationship between total folate intake and NK cell cytotoxicity (163). Women with low dietary folate intake (<233 μg/day) with daily supplement use (≤400 μg folic acid) had better NK function than those who did not take any folic acid supplements. However, women with higher dietary folate intake (≥233 μg/day) along with >400 μg/day folic acid supplement use had impaired NK cytotoxicity (163).

Folic acid supplementation may be associated with adverse health outcomes, however the exact nature and magnitude of such effects have not been clearly established. Given high intake and blood levels of folate and folic acid in North America, potential adverse health effects of folic acid supplementation need to be clearly elucidated.

2.1.8 Folate, pregnancy, and fetal development

It was previously mentioned that the RDA for women of childbearing age is 400 μg DFEs/day, and increases to 600 μg DFEs/day for pregnant women and 500 μg DFEs/day during lactation (27) to ensure the provision of folate for the rapid rate of maternal and fetal cellular growth and development (27). The aforementioned data from NHANES (169) demonstrate the wide use of prenatal vitamins, many of which contain 1 mg FA, among pregnant women in North America (109). Taking dietary intake of folate and folic acid into consideration, pregnant women most likely have a total intake of folic acid greater than 1 mg/day, the tolerable UL.

In early embryogenesis, specialized ectodermal cells form the neural plate, which bilaterally fold and fuse in the middle to create the neural tube (Figure 2.3). The neural tube
then gives rise to the central nervous system and most of the peripheral nervous system (170). This whole process is completed by 28 days following conception, approximately around the time a woman finds out that she is pregnant (171).

Figure 2.3: Neural tube development during embryogenesis. During the third week of gestation, the ectoderm, one of the three germinal layers in the developing embryo, thickens to become the neural plate. The neural tube folds inwards to form the neural groove, and the lateral sides of the groove elevate into neural folds that subsequently unite together in order to form the neural tube. When the neural tube closes bi-directionally cranially and caudally, the neural tube continues onwards to develop into the central nervous system of the fetus. The neural crest cells that lie above the neural tube and beneath the overlying skin ectoderm continue onwards to become the future peripheral nervous system of the fetus. Adapted and reprinted by permission from the publisher (Nature Publishing Group): (172) Copyright © 2005 Nature Publishing Group. All rights reserved.
For the proper closure of the neural tube, important genes that regulate developmental mechanisms must function properly. Animal studies have shown that many genes disturb key processes in the closure of the neural tube, such as normal cytoskeleton development (including Abl1 and Abl2) (173), cell proliferation or neuronal differentiation (including Brd2 and Hes1) (174), and neuroepithelial cell death (including AP2a and Bcl10) (175,176). Many others affect a number of key specific signalling pathways involved in the development of the neural tube. These pathways include: planar cell polarity, which plays a role in the initiation of neural tube closure; Sonic hedgehog signalling, which contributes to the formation of dorsolateral hinge points where the neural plate folds inwards (Fkbp8 and Ptch1) (177); BMP signalling, which prevents the formation of dorsolateral hinge points; Notch signalling, which delays neuronal differentiation until the neural tube has completely closed; and retinoid signalling, which is integral for early development (178). With failure of these pathways during the critical period in embryogenesis, the neural tube does not close and results in NTD, a congenital malformation of the central nervous system. The two most common forms of NTDs are anencephaly and spina bifida. While infants with anencephaly are either stillborn or die shortly after birth, those with spina bifida are able to survive with severe disabilities and the risk of psychosocial maladjustment (87).

Smithells et al. first identified folate deficiency in the postpartum blood of women whose offspring was born with an NTD (171). A number of epidemiological studies have demonstrated that maternal intake of folate-rich diets or folic acid supplementation reduces NTD occurrence (110,179,180). Additionally, the UK Medical Research Council conducted a double-blind, placebo-controlled, randomized multicenter clinical trial, which demonstrated that supplementation with 4 mg folic acid/day led to a threefold reduction in the risk of NTD recurrence (93). After mandatory folic acid fortification, the Center for Disease Control and Prevention (CDC) in the U. S. has reported a decrease of 26% in NTD (181), and the incidence has decreased nearly 50% in Canada (87).

The mechanisms behind folate deficiency and increased NTD risk remains unclear. In fact, the contributing factors for NTD development are most likely not limited to folate deficiency, but deficiency of other one-carbon nutrients as well (64). However, specifically for the role of folate metabolism on NTD development, environmental factors or reduced dietary intake of folate or vitamin B12 impairs remethylation of Hcy to methionine, resulting in the
intracellular accumulation of Hcy. Since the equilibrium of SAH to Hcy favors SAH, SAH accumulation occurs, leading to impaired methylation activity and consequent aberrant biological methylation reactions. These would result in the dysregulation of gene expression, protein function, and the metabolism of lipid and neurotransmitters – all of which greatly impacts the proper closure of the neural tube and cranial development (64).

Amongst the several genetic variants in the folate metabolic and one-carbon transfer pathways linked to the risk of NTDs, the MTHFR 677C>T variant, which is associated with reduced MTHFR activity and thermolabile MTHFR, has garnered most attention (64). Both children and mothers identified with this genetic variant have been observed to have an increased risk of having NTDs or have a child born with an NTD, respectively (182). Blom et al. conducted a meta-analysis of 34 studies and reported that homozygous offspring (MTHFR 677 TT) had a 90% greater risk of being born with NTDs while homozygous mothers had a 60% greater risk of giving birth to an infant with an NTD (64). The magnitude of increase was lower for individuals heterozygous for the MTHFR 677C>T variant, where heterozygous offspring had a 30% greater risk of being born with an NTD, and heterozygous mothers had a 10% greater risk of giving birth to an infant with an NTD (64).

Other genetic variants identified as possible risk factors for NTDs include methionine synthase reductase (MTRR) 66A>G, MTHFR 1298A>C, methyleneTHF dehydrogenase (MTHFD) 1958G>A and transcobalamin 776C>G (183–188). However, associations of these variants individually with NTD risk are inconsistent and small in magnitude. It is likely that more complex and multifactorial effects of genetics and environmental factors contribute to the development of NTDs.

Although the association between maternal folate deficiency and NTDs has been well supported, there are additional consequences of folate deficiency during pregnancy and birth outcomes. Due to the high expression of folate receptors in the placenta and subsequent rapid and active folate uptake, the pregnant mother is more susceptible to folate deficiency compared with the pre-pregnancy state, and has increased folate requirements (189). Although evidence generally supports the protective effect of periconceptional multivitamins containing folic acid to reduce the risk of congenital heart defects, there are a few studies that failed to detect such association (190,191). In studies of vertebrates and humans, maternal folate deficiency has also been shown to cause orofacial clefts in the offspring (192–194), and increasing folic acid intake
reduces the risk of having an affected child (195). DHFR deficiency during the gestational period of early head and facial development is associated with loss of jaw muscle and cartilage, both derivatives of the neural crest and the mesoderm, resulting in a narrower midface and malformed mouth shape (196). Wahl et al. proposed that folate deficiency may reduce the amount of tetrahydrofolate, cause DNA damage and result in reduced cell proliferation and increased cell death, impairing the normal processes of orofacial development (196). In addition to the role of DHFR, they demonstrated the effects of folate deficiency on retinoic acid, an important signalling factor in embryonic development. They showed that DHFR inhibition decreases retinoic acid receptor expression, which has previously been shown to result in median clefts in the primary palate (197). A number of studies have shown a possible link between folate deficiency and increased risk of preeclampsia (198,199), likely relating to the effects of hyperhomocysteinemia (200–202). However, a recent meta-analysis determined that there is insufficient evidence to conclude that hyperhomocysteinemia causes preeclampsia (203). While a recent cohort study in China with 10, 041 pregnant women observed a reduced risk of preeclampsia in folic acid supplement users (204), another recent study in China with 193, 554 women observed no significant reduction in the incidence of preeclampsia for women taking folic acid supplements (205).

A good indicator and predictor for fetal health is birth weight, and fetal growth restriction has been established to increase mortality and morbidity (206). Currently, there are no consistent findings of folate deficiency resulting in low birth weight, though some have observed an association between elevated maternal Hcy levels and fetal growth restriction (207–209). However, there is evidence that folic acid supplementation promotes fetal growth (210–212). A recent analysis of over 5 million births in California reported significant reduction in the incidence of preterm delivery and low- and very-low-birth weight following folic acid fortification (213).

2.1.9 Folate and offspring health risk

Maternal intake and blood levels of folate not only influence the health of the offspring at birth, but they also have implications for the offspring’s long-term health. Maternal folic acid supplementation can have neurological effects on the offspring. Barua and colleagues recently conducted a study in a mouse-model and discovered that maternal folic acid supplementation at 10x the control during gestation modulated the gene expression in the frontal cortex of pups
Although altered expression of several genes was observed, the ones that were confirmed at the translational level were down-regulation of the vestigial-like 2 gene, *Vgll2*, and up-regulation of solute carrier 17a7, *Slc17a7*. The former has been reported to impact cell fate and embryonic patterning (215). *Slc17a7* is mainly expressed in the axon terminals of neocortical neurons, playing an important role in modulating synaptic neurotransmission, and a recent study identified increased expression of this gene in schizophrenic patients (216). This group additionally proposed altered gene expression as a mechanism for the behavioural changes they observed in the pups with continued high levels of postweaning supplementation compared to those with lower levels of supplementation (214).

A number of animal studies have demonstrated that inadequate prenatal folate levels impairs proper cognitive development in the offspring (217,218). However, there are conflicting data reported from recent human studies. Tamura et al. assessed the relationship between maternal folate status in African-American women of low socioeconomic status and the cognitive development of their disadvantaged children (219). The mothers were divided into “normal” and “poor” folate status groups depending on their plasma and RBC folate concentrations, where the cut-off was 11.0 nmol/L and 430 nmol/L for plasma and RBC folate levels, respectively. The results of different cognitive tests showed that there was no significant association between maternal blood folate status and cognitive development in the children (219). In contrast, Veena et al. utilized tests of memory, attention and fluid reasoning to assess cognitive performance of 536 Indian children (220). They measured maternal micronutrient concentrations, including folate levels, for blood collected at 28-32 weeks of gestation. They reported significant improvement in cognitive performance for children born to mothers with higher blood folate concentration, although 96% of the maternal blood folate concentrations fell within the normal range (220).

In addition, gestational folic acid supplementation has been linked to the development of asthma and other allergies in the offspring (221). A few epidemiological studies have identified the link between folate intake in pregnancy and poor respiratory health in children. Whitrow et al. reported that folic acid supplement use in late pregnancy (30-34 weeks) as standalone supplements or multivitamin significantly increased the risk of asthma in the child at 3.5 years and persistent asthma at 3.5 and 5.5 years (165). Haberg et al. observed that maternal folic acid supplementation during early pregnancy was significantly associated with a small increase in wheezing in the child at 6-18 months (222). Similarly, Veeranki et al. in the US showed that the
children of women who filled folic acid-containing prescriptions during the first trimester had significantly increased odds of being diagnosed with bronchiolitis, along with greater severity, in their first year of life (223). While these studies demonstrate a significant association, recent systematic reviews have failed to support this purported relationship (224,225).

Maternal folic acid supplementation may also have impact the offspring’s risk of obesity, insulin resistance (IR) and metabolic syndrome – predictors of their long term health (31). The Pune Maternal Nutrition Study in India demonstrated that high maternal blood folate concentration was associated with increased adiposity, and the combination of high maternal blood folate and low vitamin B12 concentrations was associated with a greater risk for IR in offspring at six years of age (164). It has been proposed that the role of folate in epigenetic programming in early life influences these risk factors for the development of chronic diseases, including heart disease, stroke and diabetes later in life (226). However, suggested harmful effects of supplementation on the health of the offspring are not consistent. A recent systematic review by Xie et al. assessed the association between prenatal folic acid supplementation and obesity and IR in the offspring (227). Of the five animal studies showing a protective effect of supplementation on obesity and IR, Waterland et al. utilized the agouti (A\textsuperscript{vy}) obesity mouse model to show that transgenerational obesity was prevented by methyl donor supplementation (228). Kumar et al. used a Wistar rat model and observed a significant association between maternal folate restriction and increased visceral adiposity, cholesterol levels and risk of IR (229). Data in the five human studies were inconsistent, where protective (230) and harmful effects (164,231) of folic acid supplementation on adiposity and IR were reported, and some studies did not find any significant associations (232,233).

Maternal folic acid supplementation also appears to influence cancer risk in the offspring. Ly et al. utilized a rat model and reported that both maternal and postweaning supplementation significantly increased the incidence of mammary tumors in the offspring, accelerated the appearance and multiplicity of mammary tumors (10). In contrast, Sie et al. found that maternal, but not postweaning, supplementation decreased the incidence of colorectal cancer in the offspring by 64% in a similar rat model (15). Several epidemiologic studies have suggested, albeit not uniformly consistent, a protective effect of periconceptional dietary folate intake and folic acid supplementation on the risk of childhood cancers, including Wilms tumor, neuroblastoma, primitive neuroectodermal tumors, ependymomas and leukemia (234). A number of randomized controlled trials (RCTs) and meta-analyses have also reported a
significant protective effect of the use of folic acid-containing prenatal vitamins (235). However, the study design prohibits unequivocal conclusion that this protective effect is entirely attributed to folic acid in prenatal supplements (235).

Although the impact of maternal folate status, in particular periconceptional FA supplementation, on the health of the offspring is not clearly elucidated, the studies to date suggest that offspring’s health risk can be influenced by maternal intake of folate and FA. This begs a question as to what mechanisms might be responsible for these observed effects associated with maternal intake of folate and FA.

2.2 Folate and DNA methylation

2.2.1 Epigenetics

“Epigenetics” is defined by all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (236). These modifications include DNA methylation, histone modifications and RNA-associated silencing (237). Epigenetic changes can influence gene expression by either switching genes on or off, and consequently have functional implications for protein synthesis (237). These factors can explain why an individual’s cells all have the same DNA, yet there are specialized cells, such as neurons, hepatic, pancreatic, and inflammatory cells (236). In fact, epigenetics can give answers to why genetically identical twins exhibit some phenotypic differences, and how female mammals experience X-chromosome inactivation in order to prevent having twice the number of X-chromosome gene products as males (236). Interestingly, gene silencing is not always harmful as it is essential for preventing the expression of conserved non-genic sequences; these include transposons, pseudogenes, repetitive sequences and integrated viruses that are normally under tight regulation since they can be harmful to cells when expressed and activated (238).

2.2.2 DNA methylation

DNA methylation is important in gene expression, maintaining DNA integrity and stability, and chromatin modifications (239). In most plant, animal and fungal models, it occurs on the fifth position of cytosine, while, in mammals, it occurs on the cytosine at cytosine-guanine (CpG) dinucleotides (1). Although 60-80% of all CpG sites (28 million) are methylated in normal human DNA (240), this “global methylation” occurs where CpG density is low, including exons, noncoding regions and repeated sequences. Their methylated states do not
interfere with chromatin organization (241). Less than 10% of CpG sites densely located at the 5’ end of half of transcribed human genes are called “CpG islands” (240). They span the promoter region, untranslated region and exon 1, and are normally resistant to methylation, allowing transcription to occur (239). However, their methylation results in transcriptional silencing via the complex formed between the transcriptional repressor, methyl-CpG binding protein-2 (MBD2), and a co-repressor and histone deacetylase (HDAC) (242). The HDAC tightens nucleosomes, inactivates chromatin conformation and impairs transcription (Figure 2.4) (243).

Figure 2.4: CpG island methylation and gene silencing. Normally, DNA is packaged as nucleosomes around histones. CpG islands in the promoter region are not methylated, and modified regions of DNA [with acetylation (A) and methylation (M)] to be transcribed are in a relaxed euchromatin state, accessible to DNMTs. Methylation of CpG islands forms complexes between MBDs and HDACs. HDACs remove acetyl groups from histones, and the structure becomes compact (“heterochromatin”), impairing the binding of the transcriptional machinery. Adapted and modified by permission from the publisher (National Institute on Alcohol Abuse and Alcoholism): (244). Copyright © 2012 National Institute on Alcohol Abuse and Alcoholism.

Five members of the family of DNMTs, DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L, have been identified in mammals (245). DNMT1, DNMT3a and DNMT3b are known to transfer and maintain methyl groups from SAM to cytosine for normal mammalian development. While DNMT3a and DNMT3b are involved in de novo DNA methylation, DNMT1 is an essential maintenance enzyme (245). During embryogenesis and germ cell development, DNMT3a is thought to carry out the methylation of sequences critical for late
development or after birth, while DNMT3b may be more important for early developmental stages (246). Throughout the lifespan, DNMT1 has been shown to have a preference for hemimethylated substrates, and is responsible for restoring parental DNA methylation patterns in the newly synthesized DNA daughter strand (245). The necessity of these enzymes in mammalian growth and development has been shown through studies that demonstrated severe phenotype and embryonic lethality in mice with the inactivation of the genes encoding for the DNMTs (246,247). Although DNMT2 and DNMT3L are not believed to be cytosine methyltransferases, DNMT3L has been shown to be involved in de novo DNA methylation with DNMT3a and in transcriptional repression with HDAC1 (248).

DNA methylation involves the interplay between active methylation and active or passive demethylation. Active DNA demethylation is the removal of 5-methylcytosine (5-mC) residues on the DNA, and it has been proposed that MBDs or the reverse enzymatic reaction of DNMTs in the absence of SAM are involved (249). Passive DNA demethylation results from dysfunctional maintenance of methylation patterns, which is normally usually carried out by DNMT1 (250). The gradual loss of DNA methylation may be attributed to impaired association of DNMT1 to tether onto the replication fork (250). Recently, the Tet family of oxygenases have been discovered to shed additional light on the complex mechanism behind both active and passive DNA demethylation. It has been proposed that the Tet enzymes catalyze the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine and higher oxidative derivatives (251).

2.2.3 Epigenetic programming and modifications throughout life cycle

During mammalian development, there are periods of genome-wide reprogramming of methylation patterns in germ cells and in early embryos. Primordial germ cells (PGCs) are highly methylated and have normal imprinting patterns (252). They begin genome-wide demethylation early in development and end by embryonic day 13 to 14 (E13 to E14). At this point, PGCs are located in the gonads and most differentially methylated regions (DMRs) in imprinted genes and single-copy gene sequences are demethylated (253). It is not yet known whether the reprogramming is active or passive. When the genomes of both male and female PGCs have been demethylated, the cells enter mitotic (male) and meiotic (female) arrest. At E15 to E16, remethylation of the male germ line - single-copy gene sequences and imprinted genes – takes places (253). Remethylation of the female germ line occurs following birth during the growth of oocytes. The purposes of reprogramming in germ cells include resetting of imprints.
and removing acquired epigenetic modifications from genetic and environmental factors. Nonetheless, occasional inheritance of epigenetic information via the germ line points to the probable incomplete demethylation in the germ line or after fertilization (254).

Epigenetic reprogramming in early embryos utilizes both active and passive demethylation for the paternal and maternal genomes, respectively. Three proposed means include direct removal of the methyl group from the cytosine base (66), replacement of 5-mC with cytosine via base-excision (255), and deamination of 5-mC followed by mismatch repair. Following fertilization, the paternal genome goes through many changes in the egg cytoplasm, where the sperm chromatin is modified by replacing protamines with acetylated histones, followed by genome-wide demethylation in the early cleavage stages (256). In the paternal chromosomes, imprinted genes, such as H19 (257), and some repeat sequences are protected from demethylation. Following implantation, DNMT3a and DNMT3b are responsible for remethylation (258).
Figure 2.5: DNA methylation throughout the life cycle. [Modified from Reik et al. 2001] (A) Methylation reprogramming in the germ line. Primordial germ cells (PGCs) are demethylated early in development, and remethylation begins on E15 in male germ cells, and after birth in growing oocytes. (B) Methylation reprogramming in early embryos. The paternal genome, marked by the spermatozoan, is demethylated via an active mechanism following fertilization. The maternal genome is demethylated by a passive mechanism after the demethylation of the paternal genome begins. At implantation, marked by the formation of a blastocyst, both genomes are remethylated in embryonic (EM) and extraembryonic (EX) lineages. Adapted and reprinted by permission from the publisher (The American Association for the Advancement of Science): (259). Copyright © 2001 The American Association for the Advancement of Science. All rights reserved.

2.2.4 DNA methylation in health and disease

When there is an interference of the essential role DNA methylation plays in gene silencing and maintaining its stability, normal DNA methylation patterns can be lost, leading to impairment or loss of control of gene expression and cell growth. Changes in DNA methylation
can have implications for other epigenetic mechanisms as well, including histone modification, together leading to the development of several diseases, including cancer.

Characteristic to tumor cells is the loss of genomic stability that leads to uncontrolled cell growth (260). DNA hypomethylation, especially of repetitive regions, results in unstable alterations to the genome, and early loss of genomic methylation in cancer has been shown to correlate with severity and the metastatic potential of many tumors (261). Genome instability resulting from DNA hypomethylation is characterized by structural rearrangements of chromosomes and reactivation of transposon promoters, which interferes with normal transcription (262). Gene-specific hypomethylation additionally contributes to cancer by targeting and increasing the expression of specific genes, such as the S100 calcium binding protein A4 (S100A4) gene in colon cancer (263), the serine protease inhibitor (SERPINB5) in gastric cancer (264) and the putative oncogene γ-synuclein (SNCG) in breast and ovarian cancers (265). Robertson et al. proposed that perhaps global demethylation plays a significant role early in tumorigenesis to increase the cell’s predisposition to genomic instability, while gene-specific demethylation contributes later to stabilize the tumor cells and promote metastasis (260).

Gene-specific hypermethylation is also involved in cancer, where early hypermethylation of normally unmethylated CpG regions causes structural changes to the chromatin and transcriptional silencing (260). The hypermethylation and consequent gene-silencing have been reported in nearly all tumor types for important genes involved in cell-cycle regulation, tumor cell invasion, DNA repair, chromatin remodelling, cell signalling, transcription and apoptosis (260). Impaired expression of the specific genes results in uncontrolled tumor cell growth, genomic instability and metastatic potential (260).

Further, the role of DNA methylation in genomic imprinting also affects the development of several diseases, including cancer. As it will be explained in further detail in subsequent sections, genomic imprinting is marked by differential expression of two alleles of a gene in somatic cells of the offspring due to epigenetic modifications of a specific parental chromosome in the gamete or zygote (266). Expression of imprinted genes can be in all cells, targeted tissues or at specific developmental stages (266). A pertinent mechanism for imprinting is allelic DNA methylation localized to differentially methylated regions (DMRs) (259). DMRs contain imprinting control regions (ICRs), which regulate gene expression of the imprinted
regions. Both DMRs and ICRs can be differentially methylated, however the methylation of DMRs usually occurs during development while that of ICRs is established early in germ cells to be maintained throughout development. Methylation of imprinted regions affects the activity of CCCTC-binding factor (CTCF), an important protein that binds to highly divergent sequences and regulates the access of enhancers to the promoter region for gene expression (267). In addition to regulating imprinted gene expression, CTCF may protect DMRs from de novo methylation (268).

In cancer, LOI defects in both growth-promoter and growth-inhibitory imprinted genes can occur. First, LOI of a normally silent allele of a growth-promoting gene can lead to abnormally high expression and cell growth. For example, the imprinted IGF2/H19 locus can lead to cancer of the colon, liver, lung, ovaries and Wilms’ tumor, an embryonic kidney cancer. Insulin growth factor-2 (IGF2) is an autocrine growth factor expressed from the paternal allele, and H19 is a non-coding RNA with growth inhibiting effects expressed from the maternal allele (269). LOI for IGF2 results in increased IGF2 expression and reduced H19 expression, which gives cells a growth advantage (270). On the other hand, LOI in growth-inhibitory imprinted genes, resulting in silencing a normally active allele can also lead to uncontrolled cell growth. For example, cyclin-dependent kinase inhibitor 1C (CDKN1C), also known as p57, is a mediator of the arrest phase in the cell cycle that is expressed from the maternal allele, and LOI of CDKN1C occurs in nearly 10% of Wilms’ tumour (266).

Besides cancer, the Beckwith-Wiedemann syndrome (BWS) is a largely maternally transmitted disorder characterized by fetal and postnatal overgrowth and increased risk to embryonic tumors (260). It has been suggested that LOI silencing maternally expressed genes result in the anatomical malformations associated with BWS, and LOI that activates normally silenced IGF2 leads to tumorigenesis (271). Prader-Willi syndrome, a complex disorder affecting multiple parts of the body, is characterized by hypotonia, hyperphagia, inhibited growth, delayed development, intellectual impairment, learning disabilities and behavioural abnormalities (272). The main cause of this syndrome is the loss of paternally expressed genes, which mostly occurs through deletions in the imprinted domain (15q11-q13), but also de novo methylation of the paternal allele that results in LOI at the ICR (273). The autoimmune disease, systemic lupus erythematosus (SLE), occurs 8-10 times more frequently in females than in males (260). It is characterized by autoantibody production, synthesis of antibodies against nuclear components and defective T-cell function (274). Loss of DNA methylation is believed
to be associated with SLE through the driving force of T cells to synthesize autoantibodies. Richardson et al. reported 15-20% reduction in global methylation levels and reduced DNMT1 levels in the genomes of affected T cells (275). Hypomethylated T cells have also been shown to induce autoreactivity, where these affected T cells cause apoptosis of macrophages and reduce clearance of apoptotic material (276).

Although the pathogenesis of many diseases lies in genetic predispositions, there is a significant role of DNA methylation in changing the programming set through genetics. The implications of the involvement of DNA methylation changes through both global and gene-specific methylation, and changes to genetic imprinting on human health and disease suggest that there are mechanisms other than alterations to the DNA sequence that can profoundly impact one’s health.

2.2.5 Folate and DNA methylation

The reduction of natural folate of folic acid in fortified foods or supplements to 5-methylTHF is essential for maintaining the flow of methyl groups for the remethylation of Hcy to methionine, the substrate for SAM (23). As a universal methyl group donor, SAM is responsible for a host of biological methylation reactions, including those of DNA, ribonucleic acid (RNA), neurotransmitters, and other small molecules, phospholipids and proteins, including histones (277). Methylation of these molecules can impact gene stability and transcription, protein localization and their degradation (278). Folate, as well as other nutrients including vitamin B6, riboflavin, vitamin B12 and choline (279), are involved in one-carbon metabolism pertaining to Hcy remethylation and SAM generation and hence, DNA methylation.

2.2.5.1 Effects of folate deficiency and supplementation on DNA methylation: evidence from in vitro culture and animal studies

Some studies have reported that prolonged and severe folate deficiency is significantly correlated with global hypomethylation in the rodent liver, while moderate deficiency has no consistent effect (Table 2.1) (280,281). Some studies observed null effects (282) and others observed the opposite effect, where moderate deficiency was associated with global hypermethylation in the liver (283,284).

It has been largely observed that the colon is resistant to folate-induced effects on global DNA methylation. Folate deficiency has not been shown to significantly affect SAM:SAH
levels or genomic DNA methylation (1), although there are a few studies that reported hypomethylation (285) or hypermethylation (286). Folate supplementation also has not been shown to significantly affect SAM:SAH levels (286,287).

Wang et al. conducted an *in vitro* culture study with human neuroblastoma cells to determine the effects of folate and gene-specific methylation cell viability with respect to Alzheimer’s Disease (288). The death receptors 4 (*DR4*) gene is part of the tumor necrosis factor super family activated to induce cell apoptosis, and has previously been linked to cerebral microvascular endothelial cell apoptosis, a possible mechanism of AD development (289). They reported that folate deficient cells had significantly lower levels of promoter *DR4* methylation than folate sufficient cells (288), and suggested the possible role of folate deficiency and hypomethylation of genes, such as *DR4* in contributing to the development of Alzheimer’s Disease.
Table 2.1 Summary of associations between folate deficiency and supplementation on DNA methylation in cell culture and animal studies

**In vitro culture studies**

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Species/cell type</th>
<th>Diet/medium</th>
<th>Duration</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang et al., 2014 (288)</td>
<td>Human neuroblastoma cells</td>
<td>0 (deficient), 1, 4, 8, 16 µg/ml</td>
<td>144 hours</td>
<td>DR4 promoter methylation at 22 CpG sites</td>
<td>Deficient group: ↓methylation ($p &lt; 0.05$)</td>
</tr>
<tr>
<td>Farias et al., 2015 (290)</td>
<td>Human CRC cell lines (HCT116, LS174T, SW480)</td>
<td>0 (deficient), 4 (control), 16 folic acid mg/L (excess)</td>
<td>7 days</td>
<td>Genomic</td>
<td>HCT116, LS174T: Supplementation ↓methylation</td>
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</table>

**Animal studies**

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Species/cell type</th>
<th>Diet/medium</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keyes et al., 2007 (291)</td>
<td>Mice</td>
<td>Old (18 months), young (4 months) rats: 0, 4, 5, or 18 µmol/kg</td>
<td>20 weeks</td>
<td>Colon</td>
<td>Genomic $p16$ promoter methylation</td>
<td>Old vs. young mice: age ↓genomic methylation, ↑$p16$ methylation, Old mice: ↑global and $p16$ methylation with ↑folate ($p &lt; 0.05$)</td>
</tr>
<tr>
<td>Kotsopoulos et al., 2008 (292)</td>
<td>Rats</td>
<td>0 (deficient), 2 (control), or 8 (supplemented) mg folic acid/kg</td>
<td>1. Experimental for weeks 3-8, control until week 30 2. Control until week 8, experimental until week 30 3. Experimental until week 30</td>
<td>Liver</td>
<td>Genomic</td>
<td>Folate deficiency period ↑genomic methylation ($p &lt; 0.04$)</td>
</tr>
<tr>
<td>Study</td>
<td>Species</td>
<td>Intervention</td>
<td>Timing</td>
<td>Tissue</td>
<td>Genes</td>
<td>Results</td>
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<tr>
<td>Gao et al., 2012 (293)</td>
<td>Female mice</td>
<td>0 (deficient), 2 mg folic acid/kg</td>
<td>5 weeks prior to mating</td>
<td>Endometrium</td>
<td>Cdh1, Pgr, Esr1</td>
<td>No difference, ↑ for deficient group (p = 0.021)</td>
</tr>
</tbody>
</table>
2.2.5.2 Effects of folate deficiency and supplementation on DNA methylation: evidence from human studies

A comprehensive summary of clinical and human epidemiological studies that have examined the effects of folate deficiency and supplementation on genomic and gene-specific methylation is provided in Tables 2A, 2B and 2C.

Human clinical studies have demonstrated that folate status can alter genomic DNA methylation (Table 2A). For older women, low folate intake has been shown to reduce genomic DNA methylation in lymphocytes (134,294). For patients with colon cancer or adenomas, although there is not enough data to suggest that folate deficiency leads to reduced genomic DNA methylation, studies suggest that folate supplementation from 400 µg/day up to 10 mg/day increases genomic DNA methylation in rectal mucosa and lymphocytes (295–298). In human epidemiological studies, most have demonstrated a direct relationship between dietary and blood folate levels and genomic DNA methylation in lymphocytes and colonic mucosa (299–304) (Table 2B). This relationship appears to be more consistent for those who have had colorectal cancer, adenomas, previously resected tumors or other health concerns (305) than normal healthy individuals (306,307). The effect of dietary and blood folate concentrations on gene-specific DNA methylation remains unclear because many studies have reported a null effect (303,308–312) (Table 2C). A number of studies report increased methylation of genes associated with colorectal carcinogenesis for individuals with low dietary or blood folate levels (313–315), however others demonstrate the effect for those with high folate (316–318).

Inconsistent findings of published studies altogether highlight the complex relationship between folate and DNA methylation that has yet to be completely understood. The effects of folate status on global and gene-specific DNA methylation seem to be cell, tissue and organ-specific, as well as gene- and site-specific; the relationship depends on the form, amount and duration of folate exposure and on the stage of transformation at the time of exposure.
Table 2.2A Summary of associations between folate deficiency and supplementation on global DNA methylation in human clinical studies

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Subjects</th>
<th>Dose</th>
<th>Genomic DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenech et al., 1998 (306)</td>
<td>Normal subjects</td>
<td>2 mg/day for 12 weeks</td>
<td>Lymphocytes</td>
<td>No change</td>
</tr>
<tr>
<td>Basten et al., 2006 (319)</td>
<td>Normal subjects</td>
<td>1.2 mg/day for 12 weeks</td>
<td>Lymphocytes</td>
<td>No change</td>
</tr>
<tr>
<td>Jacob et al., 1995 (320)</td>
<td>Men</td>
<td>25 µg/day for 30 days</td>
<td>Methylation capacity (not DNA)</td>
<td>No change</td>
</tr>
<tr>
<td>Jacob et al., 1998 (134)</td>
<td>Women 49-63 years of age</td>
<td>56-111 µg/day for 9 weeks</td>
<td>Lymphocytes</td>
<td>↓120% (p &lt; 0.05)</td>
</tr>
<tr>
<td>Ingrosso et al., 2003 (321)</td>
<td>Uremic patients with hyperhomocysteinemia &amp; pre-existing hypomethylation</td>
<td>15 mg/day 5-methyTHF for 8 weeks</td>
<td>Lymphocytes</td>
<td>Restored to normal levels</td>
</tr>
<tr>
<td>Rampersaud et al., 2000 (294)</td>
<td>Women 60-85 years of age</td>
<td>118 µg/day for 7 weeks</td>
<td>Leukocytes</td>
<td>↓10% (p = 0.0012)</td>
</tr>
<tr>
<td>Jung et al., 2011 (322)</td>
<td>Men and post-menopausal women 50-70 years of age</td>
<td>800 µg/day or placebo</td>
<td>Leukocytes</td>
<td>No difference</td>
</tr>
<tr>
<td>Protiva et al., 2011 (323)</td>
<td>Healthy volunteers</td>
<td>Low folate diet for 8 weeks, then 1. 1 mg/day folic acid for last 4 weeks 2. 1 mg/day folic acid for 8 weeks</td>
<td>Colon</td>
<td>No difference</td>
</tr>
<tr>
<td>Cravo et al., 1998 (324)</td>
<td>Patients with inflammatory bowel disease</td>
<td>5 mg/day for 6 months</td>
<td>Rectal mucosa</td>
<td>No change</td>
</tr>
<tr>
<td>Cravo et al., 1994 (295)</td>
<td>Patients with colon cancer &amp; adenoma</td>
<td>10 mg/day for 6 months</td>
<td>Rectal mucosa</td>
<td>↑93% (p &lt; 0.002)</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Subjects</td>
<td>Folate source and levels</td>
<td>DNA methylation</td>
</tr>
<tr>
<td>--------------------------------</td>
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</tr>
<tr>
<td>Cravo et al., 1998 (296)</td>
<td>Cross-sectional</td>
<td>Patients with colonic adenoma</td>
<td>5 mg/day for 3 months</td>
<td>Rectal mucosa</td>
</tr>
<tr>
<td>Kim et al., 2001(297)</td>
<td>Cross-sectional</td>
<td>Patients with colonic adenoma</td>
<td>5 mg/day for 6 months, 1 year</td>
<td>Rectal mucosa</td>
</tr>
<tr>
<td>Pufulete et al., 2005 (298)</td>
<td>Cross-sectional</td>
<td>Patients with colonic adenoma</td>
<td>400 µg/day for 10 weeks</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rectal mucosa</td>
</tr>
<tr>
<td>Figueiredo et al., 2009 (325)</td>
<td>Cross-sectional</td>
<td>Patients with colonic adenoma</td>
<td>1 mg/day for 6-8 years</td>
<td>Colonic mucosa</td>
</tr>
</tbody>
</table>

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**Table 2.2B Summary of associations between folate deficiency and supplementation on global DNA methylation in human epidemiological studies**
<table>
<thead>
<tr>
<th>Study</th>
<th>Study Design</th>
<th>Participants</th>
<th>Dietary Folate Intake</th>
<th>Methylation Measurements</th>
<th>Associated Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agodi et al., 2015 (300)</td>
<td>Cross-sectional</td>
<td>Healthy non-pregnant women 13-50 years of age</td>
<td>Dietary folate intake (deficiency cutoff: 320 µg/day) + supplement use (yes/no)</td>
<td>Lymphocytes</td>
<td>Folate deficiency ↓ methylation (OR 3.6; 95% CI 1.0-12.1; ( p = 0.04 ))</td>
</tr>
<tr>
<td>Bae et al., 2014 (301)</td>
<td>Cross-sectional</td>
<td>Postmenopausal women 50-70 years of age</td>
<td>RBC folate &lt;471 (lowest tertile) vs. &gt;672 ng/mL (highest tertile) during pre-, peri-, post-fortification periods</td>
<td>Leukocytes</td>
<td>Pre-fortification: Highest tertile, ↑ methylation (( p = 0.05 ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post-fortification: Highest tertile, ↓ methylation (( p = 0.03 ))</td>
</tr>
<tr>
<td>Ono et al., 2012 (329)</td>
<td>Case-control</td>
<td>Women 20-74 years of age with invasive breast cancer</td>
<td>Dietary folate intake ≤339.9, 339.9-419.5, 419.5-521.7, &gt;521.7 µg/day</td>
<td>Leukocytes</td>
<td>↑ folate intake ↓ global methylation (( p = 0.03 ))</td>
</tr>
<tr>
<td>Pufulete et al., 2005 (298)</td>
<td>Cross-sectional</td>
<td>Patients without colon cancer or adenoma</td>
<td>Serum 18.6 nM RBC 648.1 nM Hcy 9.9 µM</td>
<td>Colon mucosa</td>
<td>( r = -0.311 ) (( p = 0.01 )) ( r = -0.356 ) (( p = 0.03 )) ( r = 0.256 ) (( p = 0.04 ))</td>
</tr>
<tr>
<td>Pufulete et al., 2003 (302)</td>
<td>Case-control</td>
<td>Patients with colon cancer or adenoma</td>
<td>Serum 12.2 vs. 18.1 nM</td>
<td>Colon mucosa</td>
<td>↓ 26% (( p = 0.009 ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ 14% (( p &lt; 0.001 ))</td>
</tr>
<tr>
<td>Al-Ghnaniem et al., 2007 (303)</td>
<td>Case-control</td>
<td>Patients with colon cancer or adenoma</td>
<td>Serum 12.3 vs. 17.9 nM</td>
<td>Colon mucosa</td>
<td>↓ 38% (( p &lt; 0.001 ))</td>
</tr>
<tr>
<td>Alonso-Aperte et al., 2008 (330)</td>
<td>Cross-sectional</td>
<td>Patients with colon cancer</td>
<td>Colon 0.49 vs. 0.95 nmol/g</td>
<td>Neoplastic vs. normal colon</td>
<td>↓ 28% (( p = 0.08 ))</td>
</tr>
<tr>
<td>Schernhammer et al., 2010 (304)</td>
<td>Prospective</td>
<td>Patients with colon cancer</td>
<td>Dietary intake ≥400 vs. &lt;200 µg/day</td>
<td>Colon cancer</td>
<td>↓ 43% risk of hypomethylation (( p = 0.05 ))</td>
</tr>
<tr>
<td>Lim et al., 2008 (331)</td>
<td>Case-control</td>
<td>Women 50-79 years of age with colonic adenoma</td>
<td>Prefortification dietary intake &lt;317 vs. ≥317 µg/1000 kcal/day Postfortification dietary intake &lt;413 vs. ≥413 DFEs</td>
<td>Leukocytes</td>
<td>Inverse association between methylation and adenoma risk, particularly in subjects with low folate intake</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Subjects</td>
<td>Folate source and levels</td>
<td>DNA methylation</td>
<td>Effect</td>
</tr>
<tr>
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<td>-----------------------------------------------</td>
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<td>-----------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Piyathilake et al., 2011 (332)</td>
<td>Prospective</td>
<td>Women positive for high-risk human papillomavirus</td>
<td>Plasma folate (ng/ml) and B12 (pg/ml) &gt;19.8 and &gt;200 vs. ≤19.8 and &lt;200</td>
<td>Peripheral blood mononuclear cells</td>
<td>↑4-fold (p = 0.04)</td>
</tr>
<tr>
<td>Fowler et al., 1998 (333)</td>
<td>Prospective</td>
<td>Patients with cervical dysplasia</td>
<td>Serum 5.2 nM</td>
<td>Cervical tissue</td>
<td>r = -0.239 (p = 0.06)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cervical tissue 1.2 ng/mg protein</td>
<td></td>
<td>r = -0.279 (p = 0.02)</td>
</tr>
<tr>
<td>Flatley et al., 2009 (311)</td>
<td>Cross-sectional</td>
<td>Patients with cervical cancer</td>
<td>RBC 685 nM</td>
<td>Cervical cells</td>
<td>No associations</td>
</tr>
<tr>
<td>Nilsson et al., 2015 (307)</td>
<td>Case-control</td>
<td>Patients with T2D and healthy controls</td>
<td>RBC (nM)</td>
<td>Liver cells</td>
<td>No correlation</td>
</tr>
<tr>
<td>Pilsner et al., 2007 (305)</td>
<td>Cross-sectional</td>
<td>Bangladeshi adults with chronic exposure to arsenic</td>
<td>Plasma &lt;9 vs. ≥9 nM</td>
<td>Leukocytes</td>
<td>↓3% (p = 0.03) r = 0.12 (p ≤ 0.05)</td>
</tr>
</tbody>
</table>

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Table 2.2C Summary of associations between folate deficiency and supplementation on gene-specific DNA methylation in human epidemiological studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Subjects</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>van Engeland et al., 2003 (313)</td>
<td>Prospective</td>
<td>Patients with colon cancer</td>
<td>Colon cancer</td>
<td>↑prevalence (p &gt; 0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>APC-1A, p14ARF, p16INK4A, hMLH1, O6-MGMT, RASSF1A</td>
<td></td>
</tr>
<tr>
<td>Al-Ghnaniem et al., 2007 (303)</td>
<td>Case-control</td>
<td>Patients with colon cancer</td>
<td>Serum 12.3 vs. 17.9 nM</td>
<td>Colon mucosa ERα, MLH1</td>
</tr>
</tbody>
</table>

Table 2.2C Summary of associations between folate deficiency and supplementation on gene-specific DNA methylation in human epidemiological studies
<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Design</th>
<th>Study Population</th>
<th>Dietary Intake or Blood Concentration</th>
<th>Cancer Type</th>
<th>Gene Associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>de Vogel et al., 2008 (308)</td>
<td>Prospective</td>
<td>Patients with colon cancer</td>
<td>Dietary intake 142.4-163.2 vs. 247-279.9 µg/day</td>
<td>Colon cancer</td>
<td>MLH1 No associations</td>
</tr>
<tr>
<td>van Guelpen et al., 2010 (316)</td>
<td>Case-control</td>
<td>Patients with colon cancer</td>
<td>Plasma ≥6.8 vs. &lt;6.8 nM</td>
<td>Colon cancer</td>
<td>CDKN2A, MLH1, CACNA1G, NEUROG1, RUNX3, SOCS1, IGF2, CRABP1 ↑ 3-fold (p &lt; 0.05)</td>
</tr>
<tr>
<td>Coppede et al., 2014 (315)</td>
<td>Prospective</td>
<td>Patients with colon cancer</td>
<td>Plasma folate &gt;4.6 vs. &lt;4.6 ng/ml</td>
<td>Resected tumor vs. adjacent healthy tissue</td>
<td>APC, MGMT, RASSF1A, CDKN2A/p16 No correlation</td>
</tr>
<tr>
<td>Kawakami et al., 2003 (317)</td>
<td>Cross-sectional</td>
<td>Patients with colon cancer</td>
<td>5,10-methylene-THF 2.95 vs. 1.53 pmol/g tissue THF 3.71 vs. 1.99 pmol/g tissue</td>
<td>Colon cancer</td>
<td>hMLH1 ↑ tissue concentration of folate intermediates for tumors with methylated promoters</td>
</tr>
<tr>
<td>Tao et al., 2009 (309)</td>
<td>Case-control</td>
<td>Women with breast cancer</td>
<td>Dietary intake 267 vs. 275-286 mg/day</td>
<td>Breast cancer</td>
<td>E-cadherin, p16, RAR-B2 No associations</td>
</tr>
<tr>
<td>Llanos et al., 2015 (310)</td>
<td>Cross-sectional</td>
<td>Women with no history of breast cancer</td>
<td>Breast folate ≤2.84 (low), &gt;2.84 ng/g (high)</td>
<td>CDKN2A/p16</td>
<td>No associations</td>
</tr>
<tr>
<td>Flatley et al., 2009 (311)</td>
<td>Cross-sectional</td>
<td>Patients with cervical cancer</td>
<td>RBC 685 nM</td>
<td>Cervical cells</td>
<td>hMLH1, DAPK, CDH1, HIC, MGMT, RARβ, GSTP1 No associations</td>
</tr>
<tr>
<td>Stidley et al., 2010 (314)</td>
<td>Prospective</td>
<td>Subjects with smoking history (≥15 years)</td>
<td>Dietary intake per 750 µg/day</td>
<td>Sputum</td>
<td>p16, MGMT, DAPK, RASSF1A, PAXα5, PAX5β, GATA-4,5 ↓16% (p = 0.04)</td>
</tr>
<tr>
<td>Study</td>
<td>Study Design</td>
<td>Patients</td>
<td>Dietary Intake</td>
<td>Cancer Type</td>
<td>Genes</td>
</tr>
<tr>
<td>-------</td>
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<td>----------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>Wang et al., 2008 (312)</td>
<td>Prospective</td>
<td>Patients with ESCC</td>
<td>Dietary intake 0 vs. 311.1 µg/day</td>
<td>ESCC</td>
<td>p16, hMLH1, MGMT</td>
</tr>
<tr>
<td>Jin et al., 2009 (318)</td>
<td>Cross-sectional</td>
<td>Patients with NSCLC</td>
<td>5,10-methylene-THF &amp; THF 3.23 pmol/g protein</td>
<td>NSCLC</td>
<td>LINE-1, CDH13, RUNX3</td>
</tr>
</tbody>
</table>

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2.2.5.3 Effects of gene variants in the folate metabolic pathway on DNA methylation

MTHFR is a key enzyme in DNA synthesis and methylation because it catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF, the methyl donor for generating methionine from homocysteine. A common variant for the MTHFR gene is MTHFR 677C→T (MTHFR C677T), which replaces the amino acid alanine with valine (334). This polymorphism results in reduced MTHFR activity, an accumulation of 5,10-methyleneTHF and subsequent homocysteinemia with changes to the intracellular content of one-carbon derivatives. This polymorphism has been associated with CVD, congenital abnormalities and negative pregnancy outcomes (335). Another less commonly explored variant is MTHFR 1298A→C (MTHFR A1298C), which replaces the amino acid glutamate with alanine. Although the evidence for the role of this genetic variant is limited, it has been suggested to affect global DNA methylation levels (334).

Studies that examined common MTHFR polymorphisms demonstrate their modulatory effect on both genomic and gene-specific methylation in healthy individuals and those with health conditions (Table 2.3). In leukocytes of healthy individuals, the MTHFR 677TT polymorphism appears to be linked to genomic DNA hypomethylation (336–339). However, the data for the effects of the common polymorphisms on DNA methylation for the colon and other tissues are inconsistent and warrant further investigation. These studies highlight the complex relationship among folate status, DNA methylation and genetic predispositions.
Table 2.3 Summary of studies that examined the interaction between the *MTHFR* C677T genotype and dietary folate intake in association with DNA methylation

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Design</th>
<th>Subjects</th>
<th>Folate dose, source and levels</th>
<th>Duration</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stern et al., 2000 (336)</td>
<td>Cross-sectional</td>
<td>Healthy volunteers with <em>MTHFR</em> 677TT/CC</td>
<td>RBC 2.3 nmol/g Hb</td>
<td>NA</td>
<td>Leukocytes, genomic</td>
<td>$r = -0.738 \ (p = 0.02)$ with <em>MTHFR</em> 677TT</td>
</tr>
<tr>
<td>Shelnutt et al., 2004 (337)</td>
<td>Clinical</td>
<td>Normal female subjects 20-30 years of age with <em>MTHFR</em> 677TT or CC</td>
<td>115 µg/day, then 400 µg/day</td>
<td>7 weeks, 7 weeks</td>
<td>Leukocytes, genomic</td>
<td>↓5% ($p = 0.08$) after depletion, then ↑8% ($p = 0.04$) with <em>MTHFR</em> 677TT</td>
</tr>
<tr>
<td>Quinlivan et al., 2005 (340)</td>
<td>Clinical</td>
<td>Normal female subjects with <em>MTHFR</em> 677TT or CC</td>
<td>[$^{15}$C$_3$]methionine infusion and folate depletion (115±20 µg DFE/day)</td>
<td>7 weeks</td>
<td>Monocytes methyldeoxycytidine 1-carbon enrichment</td>
<td>↑ ($p = 0.012$) with folate depletion, but no effect by genotype</td>
</tr>
<tr>
<td>Axume et al., 2007 (338)</td>
<td>Clinical</td>
<td>Normal female subjects with <em>MTHFR</em> 677RR, CT, CC</td>
<td>135 µg/day, then 400 or 800 µg/day</td>
<td>7 weeks, 7 weeks</td>
<td>Leukocytes, genomic</td>
<td>↓4% ($p &lt; 0.05$) with <em>MTHFR</em> 677TT</td>
</tr>
<tr>
<td>La Merrill et al., 2011 (341)</td>
<td>Prospective</td>
<td>Pregnant women</td>
<td>Dietary intake (mg/day)</td>
<td>NA</td>
<td>Blood, genomic</td>
<td>No associations with genotype</td>
</tr>
<tr>
<td>Pizzolo et al., 2011 (342)</td>
<td>Clinical</td>
<td>Hyperhomocysteinemic men with <em>MTHFR</em> 677TT</td>
<td>5 mg/day</td>
<td>8 weeks</td>
<td>Peripheral blood mononuclear cells, genomic</td>
<td>No change</td>
</tr>
<tr>
<td>Authors</td>
<td>Study Design</td>
<td>Participants</td>
<td>Measures</td>
<td>Results</td>
<td></td>
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<td>------------------</td>
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</tr>
<tr>
<td>Friso et al., 2002 (339)</td>
<td>Cross-sectional</td>
<td>Patients with and without CAD with MTHFR 677TT or CC</td>
<td>Plasma &lt;12 vs. ≥12 nM RBC &lt;1.1 vs. ≥1.1 nmol/g Hb</td>
<td>↓61% (p &lt; 0.0001) in subjects with MTHFR 677TT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kraunz et al., 2006 (343)</td>
<td>Case-control</td>
<td>Patients with HNSCC</td>
<td>Dietary intake low vs. high</td>
<td>HNSCC p16&lt;sup&gt;INK4A&lt;/sup&gt; ↑2.3-fold (p ≤ 0.05) with MTHFR 677TT increasing risk of methylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsiung et al., 2007 (344)</td>
<td>Case-control</td>
<td>Patients with HNSCC</td>
<td>Dietary intake low vs. high</td>
<td>↓methylation in low folate + MTHFR 677T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Curtin et al., 2007 (345)</td>
<td>Case-control</td>
<td>Patients with colon cancer</td>
<td>Dietary intake &lt;135-152 (low), 135-201 (medium), &gt;180-201 (high) μg/1000kcal/day</td>
<td>Colon cancer p16, MLH1, MINT-1,-2,-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Van den Donk et al., 2007 (346)</td>
<td>Case-control</td>
<td>Patients with colorectal adenoma and MTHFR 677TT</td>
<td>Dietary intake &gt;212 vs. &lt;183 μg/day</td>
<td>↓28-70% (p &gt; 0.05)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓63% (p = 0.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓22% (p = 0.06)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Population</td>
<td>Serum folate</td>
<td>CRC tumors</td>
<td>Genomes</td>
<td>Notes</td>
</tr>
<tr>
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</tr>
<tr>
<td>Mokarram et al., 2008 (347)</td>
<td>Prospective</td>
<td>Patients with sporadic CRC</td>
<td>Serum &gt;5.5 vs. &lt;5.5 ng/ml</td>
<td>NA</td>
<td>p16, hMLH1, hMSH2</td>
<td>↑ methylated tumors in high folate group in those with MTHFR 677CT (p = 0.01) and CT/TT (p = 0.002)</td>
</tr>
<tr>
<td>Hanks et al., 2013 (348)</td>
<td>Cross-sectional</td>
<td>Men and women (&gt;19 years of age) without colorectal neoplasia</td>
<td>Serum folate (nM) 25.4</td>
<td>NA</td>
<td>Colon</td>
<td>No associations with genotype</td>
</tr>
<tr>
<td>Liu et al., 2013 (334)</td>
<td>Case-control</td>
<td>Patients with glioma</td>
<td>Serum folate: &lt;2.34 ng/ml vs. 2.34-17.56 ng/ml</td>
<td>NA</td>
<td>Blood</td>
<td>Lower frequency of methylated MGMT (p &lt; 0.001) in subjects with MTHFR 677TT and low serum folate (&lt;2.34 ng/ml)</td>
</tr>
</tbody>
</table>

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2.2.5.4 Effects of maternal methyl group supplementation on DNA methylation in animal studies

In fetal development, the embryo primarily depends on the mother to fulfill its nutritional requirements and draws its supply of methyl donors from the intrauterine environment for DNA synthesis and methylation. Many studies have examined methylation at metastable epialleles, which are variably expressed alleles in individuals of identical genotype as a result of epigenetic modifications established early in development (349). Although there is tight regulation of tissue-specific CpG methylation patterns at most genomic loci, the establishment of methylation patterns at these metastable epialleles are stochastic and can result in significant differences in DNA methylation, gene expression and phenotypes among individuals (14). The following is a summary of some relevant key studies and recent developments in this topic.

In 1998, Wolff et al. were the first to observe the effects of maternal supplementation with methyl donors in heterozygous viable yellow agouti ($A_vy/a$) mice on the DNA methylation at the $A_vy$ metastable epiallele (13). The agouti gene, when expressed, induces melanocytes within hair follicles to switch their pigment production from eumelanin (black) to phaeomelanin (yellow). In addition to the change in hair pigmentation, the agouti protein also impairs satiety signalling in the hypothalamus, thus the $A_vy/a$ mice are more prone to obesity (350). The expression of this gene can be modulated by methylation of the intracisternal A-particle long tandem repeats, which contains promoters and enhancers (350). In this study, the supplemented diets consisted of many methyl donors, including choline, betaine, folic acid and vitamin $B_{12}$. Increasing levels of supplementation resulted in increased levels of DNA methylation, and changes to the coat colour of the mice were evident (13). For those with higher levels of DNA methylation, the coat color was browner whereas, for those with lower levels methylation, their color was closer to the agouti yellow. The results suggested that maternal diet supplemented with methyl donors may increase DNA methylation at the $A_vy$ metastable epiallele, and may consequently impair agouti gene expression. In 2003, Waterland et al. also reported similar results from their study, in which female mice were randomly assigned to either a methyl donor supplemented (choline, betaine, folic acid and vitamin $B_{12}$) or standard diet for two weeks before mating (351), and the effects on coat color and $A_vy$ methylation persisted to adulthood, suggesting that the effects of methyl supplementation in utero may have an irreversible and lasting impact for the offspring.
Waterland et al. reported similar effects of maternal methyl donor on epigenetic modulations for the *axin fused* (*AxinFu*) mouse (14). The intracisternal-A particle within intron 6 of the *Axin* gene induces its expression, which results in a characteristic kink in the mouse’s tail as this gene is involved in axial pattern signaling (352). Hypermethylation of *AxinFu* silences the gene, and there is no tail kink. They reported an association between maternal methyl donor supplementation (choline, betaine, folic acid and vitamin B_{12}) for two weeks prior to mating and an increase in the level of methylation at 6 CpG sites of the *AxinFu* metastable epiallele of the offspring, and a decreased degree of tail kink (14).

With the findings that maternal dietary supplementation of methyl donors can modulate DNA methylation patterns at unique genomic loci and lead to phenotypic changes in the offspring, other studies have examined the link to the disease development. Schaible et al. conducted a mouse model study and reported that offspring born to methyl donor supplemented (folic acid, vitamin B_{12}, betaine, choline) dams had greater susceptibility to induced colitis and DNA methylation changes in their colonic mucosa (353). On the other hand, Delaney et al. reported the protective effect of maternal methyl donor supplementation on atherosclerotic development in the offspring mouse (290). Female Apolipoprotein E knockout (ApoE^{-/-}) mice with propensity to spontaneously develop atherosclerosis were given either a control or methyl-donor supplemented diet (methionine, folic acid, vitamin B_{12}, zinc, betaine and choline) for three weeks prior to mating (290). They observed that the offspring born to supplemented dams had significantly fewer atherosclerotic lesions in the aorta than those born to control dams (*p* < 0.05), and significantly higher methylation levels in their splenic T cells up to 34 weeks of age (*p* ≤ 0.01) (290). The persistent methylation patterns and previously established association between autoimmunity and T cell DNA hypomethylation (354) suggest that maternal methyl donor supplementation may protect the offspring from atherosclerosis by T cell DNA hypermethylation.

In summary, although increased levels of methyl donors in diet or as supplements are suggested for women in their reproductive age, the studies above mark the need for further consideration of both the protective and potentially harmful and irreversible effects of maternal methyl donor supplementation on the offspring’s health even into adulthood.
2.2.5.5. Effects of maternal folate status and folic acid supplementation on DNA methylation (global and gene specific) in animal studies and human studies

It has been established that adequate maternal folate supply is necessary to meet the demands of the fetus for proper neural tube closure, however, what is not yet fully understood are the effects of maternal folate supplementation on the DNA methylation patterns of the offspring and their implications for the health of the offspring throughout life. A comprehensive summary of animal and human studies examining these effects is provided in Table 2.4.

It is difficult to summarize the effects reported in these studies because they vary in study design and utilized different models. Not only are the data inconsistent, but the inconsistencies lie for each tissue examined. Nonetheless, in general, there is sufficient data to suggest that DNA methylation of the offspring is modulated by intrauterine exposure to folate alone or in combination with other methyl donors. These methylation changes can affect normal development of the offspring and have implications for their health later in life. Since folate levels in cord blood are three-fold of that in the mother, it is important to continue to investigate the possible implications of a heightened intrauterine environment to folate on the offspring.
Table 2.4 Summary of studies that examined *in utero* and perinatal folate supply on DNA methylation in the offspring

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Species</th>
<th>Folate source, levels</th>
<th>Duration</th>
<th>Tissue</th>
<th>DNA methylation</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>McKay et al., 2011 (11)</td>
<td>Mice</td>
<td>0.4 vs. 2 mg folic acid/kg</td>
<td>4 weeks before mating + during pregnancy</td>
<td>Colon</td>
<td>Sk394a Esr1, Igf2</td>
<td>↓ 3.4% (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>McKay et al., 2011 (355)</td>
<td>Mice</td>
<td>0.4 vs. 2 or 8 mg folic acid/kg</td>
<td>Mating, pregnancy, lactation</td>
<td>Small intestine</td>
<td>Genomic</td>
<td>↓ (<em>p</em> &lt; 0.01)</td>
</tr>
<tr>
<td>McKay et al., 2011 (356)</td>
<td><em>Apc</em>&lt;sup&gt;−/Min&lt;/sup&gt; and WT mice</td>
<td>2 vs. 0.4 mg folic acid/kg</td>
<td>Pregnancy + lactation</td>
<td>Small intestine</td>
<td>Igf2, Apc p53</td>
<td>No differences</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Significant decrease in adult mice (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>Sie et al., 2011 (12)</td>
<td>Rats</td>
<td>5 vs. 2 mg folic acid/kg</td>
<td>3 weeks before mating through pregnancy + lactation</td>
<td>Colorectum at weaning</td>
<td>Genomic</td>
<td>↑ 3% (<em>p</em> = 0.007)</td>
</tr>
<tr>
<td>Sie et al., 2013 (15)</td>
<td>Rats</td>
<td>5 vs. 2 mg folic acid/kg</td>
<td>3 weeks before mating through pregnancy + lactation</td>
<td>Liver at weaning</td>
<td>Genomic</td>
<td>↓ 25% (<em>p</em> &lt; 0.001)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>↓(<em>p</em> &lt; 0.05)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>No differences</td>
</tr>
<tr>
<td>Finnell et al., 2002 (357)</td>
<td>Mice</td>
<td>25 mg/kg/day folic acid by gavage</td>
<td>2 weeks before mating to D15.5 of gestation</td>
<td>Liver Brain</td>
<td>Genomic</td>
<td>↓ 4-fold (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>↓ 2-fold (<em>p</em> &lt; 0.05)</td>
</tr>
<tr>
<td>Study</td>
<td>Species</td>
<td>Treatment Details</td>
<td>Duration</td>
<td>Tissue</td>
<td>Type</td>
<td>Effect Description</td>
</tr>
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<tr>
<td>Maloney et al., 2007 (358)</td>
<td>Rats</td>
<td>1. Control</td>
<td>2 weeks before mating to D21 of gestation</td>
<td>Liver</td>
<td>Genomic</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Folate -/-</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3. Folate -/-, low methionine</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>4. Folate -/-, low choline</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>5. Folate -/-, low methionine and choline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cho et al., 2013 (359)</td>
<td>Rats</td>
<td>20 vs. 2 mg folic acid/kg</td>
<td>Pregnancy + lactation + postweaning</td>
<td>Brain</td>
<td>Genomic</td>
<td>No difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ during weaning</td>
</tr>
<tr>
<td>Kim et al., 2009 (360)</td>
<td>Rats</td>
<td>1. 8 mg folic acid/kg</td>
<td>4 weeks before D20 of gestation</td>
<td>Placenta</td>
<td>Genomic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. 8 mg folic acid/kg, 0.3% Hcy</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>3. 0.3% Hcy</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Kulkami et al., 2011 (361)</td>
<td>Rats</td>
<td>8 mg folic acid/kg and B12 -/-</td>
<td>Pregnancy</td>
<td>Placenta</td>
<td>Genomic</td>
<td>↓ (p &lt; 0.05)</td>
</tr>
<tr>
<td>Ly et al., 2011 (10)</td>
<td>Rats</td>
<td>5 vs. 2 mg folic acid/kg</td>
<td>3 weeks before mating through pregnancy + lactation</td>
<td>Mammary</td>
<td>Genomic</td>
<td>↓7% (p = 0.03)</td>
</tr>
<tr>
<td>Ciappio et al., 2011 (362)</td>
<td>Mice</td>
<td>Vitamin B 8 vs. 2 mg folic acid/kg</td>
<td>4 weeks before mating through pregnancy + lactation</td>
<td>Small intestine mucosa</td>
<td>Genomic</td>
<td>↓3% (p = 0.07)</td>
</tr>
<tr>
<td>Study</td>
<td>Species</td>
<td>Intervention</td>
<td>Timeframe</td>
<td>Tissue</td>
<td>CpG sites</td>
<td>Status of CpG sites</td>
</tr>
<tr>
<td>-------------------------------</td>
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</tr>
<tr>
<td>Sinclair et al., 2007 (363)</td>
<td>Sheep</td>
<td>Vitamin B12 and folate deficient vs. control</td>
<td>8 weeks before 6 days after conception</td>
<td>Liver</td>
<td>1400 CpG sites</td>
<td>Altered status at 4% of CpG sites (p &lt; 0.001)</td>
</tr>
<tr>
<td>Fryer et al., 2009 (364)</td>
<td>Humans</td>
<td>Folic acid 400 µg/day</td>
<td>Pregnancy</td>
<td>Cord blood</td>
<td>Genomic</td>
<td>r = 0.364 (p = 0.08)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cord serum 15.8 µM</td>
<td></td>
<td></td>
<td></td>
<td>r = 0.209 (p &gt; 0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cord Hcy 10.8 µM</td>
<td></td>
<td></td>
<td></td>
<td>r = -0.688 (p = 0.001)</td>
</tr>
<tr>
<td>Ba et al., 2011 (114)</td>
<td>Humans</td>
<td>Cord serum 7.29 ng/ml</td>
<td>NA</td>
<td>Cord blood</td>
<td>IGF2 promoter 2, 3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maternal serum 2.29 ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steegers-Theunissen et al., 2009 (365)</td>
<td>Humans</td>
<td>Folic acid supplement 400 µg/day</td>
<td>Periconceptional</td>
<td>Whole blood at 17 months</td>
<td>IGF2</td>
<td>↑4.5% (p = 0.014)</td>
</tr>
<tr>
<td>Hoyo et al., 2011 (366)</td>
<td>Humans</td>
<td>Folic acid users vs. nonusers during pregnancy</td>
<td>NA</td>
<td>Cord blood</td>
<td>IGF2 DMR H19 DMR</td>
<td>No associations</td>
</tr>
<tr>
<td>Hoyo et al., 2014 (367)</td>
<td>Humans</td>
<td>Maternal RBC 217.8 ng/ml</td>
<td>NA</td>
<td>Cord blood</td>
<td>PLAGL1, SGCE, DLK1/MEG3, IGF2/H19</td>
<td>Significant association (p &lt; 0.05)</td>
</tr>
<tr>
<td>Van Mil et al., 2014 (368)</td>
<td>Humans</td>
<td>Maternal plasma folate, folic acid supplement use</td>
<td>NA</td>
<td>Cord blood</td>
<td>5-HTT, DRD4, IGF2, H19, KCNQ1OT1, CCNL1/LEKR1, MTHFR, NR3CI</td>
<td>No associations</td>
</tr>
<tr>
<td>Authors</td>
<td>Species</td>
<td>Condition</td>
<td>Value</td>
<td>Gene</td>
<td>Effect</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
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<td></td>
</tr>
<tr>
<td>Amarasekera et al., 2014 (369)</td>
<td>Humans</td>
<td>Maternal serum</td>
<td>74.59 vs. 16.8 nmol/L</td>
<td>NA</td>
<td>Cord CD4+ T cells and APCs</td>
<td></td>
</tr>
</tbody>
</table>

*ZFP57* ↓ in high folate group (*p* < 0.05)

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2.3 Paternal folate status on DNA methylation and health risk in the offspring

2.3.1 Spermatogenesis

The transformation of germ cells into spermatozoa in the seminiferous tubule of the testis over a period of time is called “spermatogenesis” (370) (Figure 2.6).

**Figure 2.6: Spermatogenesis.** The process of spermatogenesis in the seminiferous tubule. Primordial germ cells, which are the stem cell population of gametogenesis, reside in the basement membrane and undergo mitosis to proliferate. Over the course of 64 days, the cells subsequently undergo meiotic divisions in order to differentiate and mature into secondary spermatocytes and spermatids that further migrate towards the lumen. Adapted and reprinted by permission from the publisher (The Royal Society): (371) Copyright © 2002 The Royal Society. All rights reserved.

The structure of the tubules consist of a seminiferous epithelium lining and a fluid-filled lumen, where the fully developed spermatozoa enter (370). There are three phases in spermatogenesis – proliferation, meiosis and differentiation. In proliferation, spermatogonia, the most immature cells, reside at the basement membrane of the seminiferous epithelium and multiply through mitosis. This mitotic division is continuous and repetitive in order to maintain the germinal epithelium. Spermatogonia produce both stem cells and committed cells that will...
eventually become spermatozoa. The final mitotic division of the spermatogonia produces the preleptotene spermatocyte, which moves away from the tubule base and across the Sertoli-Sertoli junction (370). During the second phase, meiosis, spermatocytes increase their DNA content and go through two divisions in order to produce four haploid germ cells. Meiosis begins with DNA synthesis occurring in preleptotene spermatocytes, and chromosomes unravel during prophase of the first division. This step, which also consists of homologous chromosomes pairing up and forming the synaptonemal complex, takes about 3 weeks (370). Thereafter, in what are now slightly larger pachytene spermatocytes, genetic recombination via cross-over between paired chromosomes occurs. The synaptonemal complexes separate, and the chromosomes spread apart within the nucleus. Finally, in diakinesis, the disappearance of the nuclear envelope and the condensation of the chromosomes marks the formation of small secondary diploid spermatocytes. Their rapid second meiotic division produces even smaller haploid cells that are called “round spermatids” (370). In the final stage of spermatogenesis, called “differentiation” or “spermiogenesis” the haploid germ cells go through final changes to become a mature sperm cell. The first modification involves the elongation of the nucleus and chromatin condensation, resulting in unique species-specific shapes. Additionally, the Golgi apparatus produces granules over the nucleus that eventually become the acrosome, which holds the hydrolytic enzymes necessary in fertilization. Finally, the germ cell discards excess cytoplasm and produces a long mitochondria-containing tail, which mobilizes the sperm cell.

2.3.2 Epigenetic processes in spermatogenesis

DNA methylation

De novo methylation and demethylation set unique methylation patterns in testicular germ cells before meiosis, until up to early prophase I (372). These DNA methylation patterns are mostly established by the end of the pachytene spermatocyte step. In these cells, methylation occurs mostly at non-CpG island sequences in distinct loci and repetitive sequences, within known genes or between genes (373). During spermatogenesis, the methylation patterns of specific genes may vary in accordance to the various stages, and may not be reflected in their expression.

Genetic imprinting

Genetic imprints are sex-specific epigenetic patterns on discrete loci that result in monoallelic gene expression (374). As previously mentioned, DMRs are methylated CpG
islands of imprinted loci that consist of short indirect repeats or larger unit repeats; these regions dictate gene expression (375). The best-characterized paternally imprinted gene is the IGF2/H19 locus, which show reciprocal expression of the paternal IGF2 and maternal H19 loci (376). Insulator proteins, including CTCF and Brother of the regulator of the imprinted site (BORIS), have been suggested to also regulate gene transcription (267). CTCF is known to bind to DMRs in order to prevent the interaction between promoter and enhancer, and it has been suggested that BORIS interacts with demethylases to remove imprints and with methylases for de novo methylation (267).

Epigenetic reprogramming during spermatogenesis

Immediately after fertilization, genomic methylation patterns of primordial germ cells are erased and re-established for totipotency. Before the cells reach the gonadal ridge, most epigenetic marks set during spermatogenesis are removed. Thus, de novo methylation during embryonic development and spermatogenesis can occur in a sex-specific manner (377).

Nuclear protein transitioning

For efficient transfer of paternal DNA to the oocyte, male germ cells are specifically packaged smaller than somatic cells. Replacing histones with transition proteins first, then with protamines, achieves this compact package since protamines are very basic nuclear proteins that neutralize the negative charge of DNA (378). Histone H4 is hyperacetylated prior to histone removal from haploid spermatids in order to lower the histone’s affinity to DNA, allowing easy exchange of histones for protamines. At completion of the exchange, about 15% of the histones still remain (379). These histones are still important in male gametogenesis, however the specific structure and interaction between protamines and DNA are not fully understood yet (374).

Histone variants

Although at a low frequency, it has been shown that testis-specific histone variants crucial in spermatozoa development and maintenance are retained in the mature sperm chromatin. The most important histones include H2A, H2B, H3 and H4, and variants of H2A and H2B have been proposed to play a role in reprogramming pericentric heterochromatic regions during spermatogenesis (379). As mentioned before, H4 hyperacetylation facilitates
easy nuclear protein transitioning by lowering its affinity to DNA. Other retained histones include HILSI and HIt2, both of which may play a role in chromatin remodelling (380).

2.3.3 DNA methylation in sperm and the offspring’s health

DNA methylation of certain genes has been shown to affect semen conditions and male factor infertility (374). Houshdaran et al. have shown that hypermethylation of PAX8, NTF3, SFN, HRAS and the repetitive element Satellite 2 is associated with poor semen concentration, motility and morphology. They suggested that hypermethylation resulted from an error in the removal of established methylation patterns, not from improper de novo methylation (381). Other studies have linked the Jumonji C-terminal containing histone demethylase 2A (JHMD2A), involved in the regulation of chromatin condensation in sperm packaging, to male infertility, obesity and spermatogenesis (382). Additionally, Khazamipour et al. showed that 53% of men with non-obstructive azoospermia had hypermethylation of the MTHFR promoter in testes, while none of the men with obstructive azoospermia had hypermethylation (383). Finally, Wu et al. demonstrated a significant association between hypermethylation of MTHFR gene promoter in sperm and male infertility (384).

2.3.3.1 The effect of paternal nutritional factors on offspring’s health

One of the earliest studies examining paternal contribution to the health of their offspring was conducted by Bygren et al. in Northern Sweden with subjects born in 1890, 1905 and 1920, followed until death or 1995 (385). Information regarding their parents’ and grandparents’ access to food before their “slow growth period”, before their pre-pubertal peak, was taken from historical records of harvests and food prices. They reported that the offspring’s mortality from CVD decreased with fathers’ low access to food, and mortality from diabetes increased with grandfathers’ greater access to food. This study was one of the seminal works suggesting transgenerational effects of diet on the offspring’s health through the male line.

A number of studies have shown that paternal obesity negatively impacts fertility and pregnancy outcomes. Kort et al. analyzed semen samples from men with normal (20-24 kg/m²), overweight (25-30 kg/m²) and obese (>30 kg/m²) body mass index (BMI) values (386). They found significant differences in sperm motility and chromatin integrity among men from the different groups, where men with normal BMI had the greatest number of normal motile sperm cells, and obese men had the lowest ($p < 0.05$) (386). They additionally reported reduced sperm integrity in the overweight and obese men when compared to the normal group ($p < 0.05$). Since
sperm structure has previously been shown to affect fertility (387), these results suggest that paternal diet and habits may negatively impact pregnancy outcomes. The data are consistent with previous studies (388,389), and a study by Fejes et al. reported a negative correlation between male obesity and motile sperm count (390). Other studies have shown additional links between paternal obesity and sperm concentration (391), and reduced volume of ejaculate (392). However, some have found no significant associations between obesity and abnormal sperm morphology (389,392,393). Interestingly, smoking, alcohol use and caffeine intake have all been shown to increase sperm aneuploidy - an abnormal number of chromosomes resulting from an error in cell division and a common cause of poor reproductive outcomes and negative health outcomes in the offspring (394).

In addition to its effects on sperm, higher paternal BMI has been shown to be significantly associated with reduced implantation, pregnancy rates and live births in couples undergoing assisted reproductive technology in a fertility clinic (395). Similarly, zygotes of obese male mice on fed a 7-week high fat diet (HFD) prior to mating had impaired blastocyst formation, implantation and reduced live fetuses (21). Ghanayem et al. examined the effects of paternal obesity on both sperm motility and reproductive outcomes (396). In genetically identical male mice, the ones fed a diet containing lard had sperm with lower motility than the lean mice (396). Female mice with obese mating partners had significantly fewer plug formations (indicator of successful mating) ($p < 0.01$) and pregnancies ($p < 0.0001$) compared to those with lean partners (396). Findings from a similar recent study using a rat model suggest that a HFD may lead to such impairments of male reproductive function by inducing a pre-diabetic state and modulating testicular metabolism (22).

Furthermore, paternal interval fasting with a duration of 24 hours during the 4 week period prior to mating significantly decreased fasting serum glucose levels in the 10 week-old offspring by 12% ($p < 0.01$) (397). This magnitude of change may be biologically meaningful because the risk of several cancers has been linked to differences in serum glucose of around 15% (398). Ng et al. reported that female offspring with sires fed chronic HFD of 10 weeks exhibited significantly impaired glucose tolerance and insulin secretion compared to the control group ($p < 0.05$) (18). These studies suggest that paternal diet may have implications beyond fertility and pregnancy, to metabolic consequences of the offspring.
Human studies have shown that indicators of offspring lipid status in the blood may independently reflect those of the father. Ohlund et al. reported a significant association between paternal and offspring total cholesterol levels from 6 months to 4 years of age ($p \leq 0.05$) (399), and Marcovecchio et al. have shown significant associations between paternal and offspring total cholesterol, triglycerides, high density lipoprotein C, low density lipoprotein C and non-high density lipoprotein C ($p < 0.0001$) (400). Both studies show that the previously described effects of paternal HFD on offspring metabolic function may be reflected in their blood lipid profiles.

Additional studies have examined the effects of indicators of paternal nutrition on the cardiovascular function of the offspring. A large cross-sectional study using data from more than 14 0000 families participating in the Health Study of Nord Tronsted County in Norway examined parental influences on their offspring’s CVD risk. They reported significant associations between offspring low birth weight, a risk factor for CVD, and increased paternal blood pressure ($p = 0.001$), BMI ($p = 0.001$), glucose and lipid levels ($p < 0.001$) (401). While Myklestad et al. utilized parameters that reflect paternal nutrition (401), Watkins et al. used a mouse model to observe the effects of paternal low protein diet (LPD) on the cardiovascular and metabolic functions of the offspring (402). They found that paternal LPD is significantly associated with lower blood pressure and greater heart rate ($p < 0.05$). They also reported impaired glucose intolerance and elevated adiposity in the LPD offspring (402).

Furthermore, paternal nutrition containing B vitamins has been examined for its effects on offspring’s health. Ratan et al. conducted a study to observe the effects of parental folate, vitamin B$_{12}$ and choline levels in whole blood, RBC and serum on those of neonates with NTDs, other congenital anomalies and those without anomalies (403). They reported significantly reduced blood folate and vitamin B$_{12}$ levels, and increased plasma homocysteine levels in fathers of neonates with NTDs compared to the other groups ($p = 0.01$) (403). However, high paternal plasma homocysteine level was the only independent risk factor for NTDs in the offspring. Young et al. analyzed sperm samples from healthy non-smoking men, and reported that folate intake had a significant negative association with the frequency of aneuploidy in sperm ($p \leq 0.04$), and there was no association between zinc or antioxidant intake and aneuploidy (404). Finally, a recent case-control study by Bailey et al. examined the relationship between preconceptional paternal dietary intake of folate, vitamin B$_{6}$ and B$_{12}$ and the risk of childhood acute lymphoblastic leukemia (405). Although they did not detect consistent
associations between the paternal folate or vitamin $B_6$ intake and the risk of leukemia, they did report a nearly significant association for high paternal vitamin $B_{12}$ intake and increased offspring risk ($p = 0.06$) (405). Finally, Sabet et al. used $Apc1638N$ mice prone to developing intestinal tumors in order to examine the effects of paternal diet either deficient of or supplemented with B-vitamins, including folate, for 8 weeks prior to mating (406). They reported an increase in tumor volume for the female offspring that corresponded with increasing paternal diet levels of vitamin B and elevated hepatic triglycerides and cholesterol for those with supplemented fathers. All offspring to the supplemented fathers had altered expression of genes involved in lipid metabolism, and many genes were differentially methylated in the sperm.

Paternal nutrition and its indicators have been attributed to important health consequences in the offspring. The effects have been evident as early as during the parental mating period and throughout pregnancy, further into the offspring’s birth and adulthood. Although paternal contribution to the offspring was previously believed to be null in comparison to that of the mother, the studies above show that consideration and continued examination of the effects of paternal nutrition and lifestyle factors are much warranted.

2.3.3.2 The effect of paternal nutritional factors on offspring’s epigenetics including DNA methylation

Although the evidence in literature is limited, epigenetic mechanisms have been suggested to play a mechanistic role in the relationship between paternal nutrition and the offspring’s health. Interestingly, most of the studies have addressed the epigenetic effects of paternal HFD on the offspring. In the rat model study by Ng et al. that showed a significant association between paternal obesity and impaired metabolic functions of the pancreas in the offspring (18), they additionally reported 77 genes with altered expression levels in the HFD group compared to the control ($p < 0.001$). Out of these genes, the interleukin 13 receptor subunit $\alpha_2$ ($Il13ra2$) gene was significantly hypomethylated in the offspring of HFD fathers compared to those from the control group ($p < 0.001$) (18). Because this gene is expressed in pancreatic cell lines with the function of regulating growth and invasion of these cells (407), the results suggest that paternal HFD may influence pancreatic islet function through epigenetic mechanisms. In a mouse study conducted by Binder et al., male mice received a HFD or control diet for 10 weeks prior to sperm collection for in vitro fertilization and embryo transfer (408).
They reported increased global DNA methylation levels in the female placenta, but not in male placenta, suggesting potential sex-specific effects on offspring global DNA methylation.

Inconsistent data suggest that the epigenetic effects of paternal HFD on the offspring reflect similar complexity of the effects of maternal diet. As previously mentioned, *IGF2* is an imprinted gene that encodes an important active growth factor throughout embryogenesis and fetal development (409). Methylation of *IGF2* and *H19* DMRs regulate imprinting on the *IGF2* allele, and only the paternal allele is normally transcribed in humans. Soubry et al. demonstrated that paternal BMI had a significant negative association with *IGF2* DMR methylation in cord blood leukocytes (*p* < 0.01), but no significant association with the methylation of *H19* DMR (410). More recently, they showed that preconceptional paternal obesity was significantly associated with reduced methylation at the mesoderm-specific transcript (*MEST*) gene, *PEG3* (*p* < 0.01) and neuronatin (*NNAT*) gene (*p* < 0.05) in cord blood leukocytes (411). However, in a mouse model study examining the effects of HFD on the methylation of ICRs of sperm, they found no significant differences between the HFD and control group in the ICR methylation of *H19*, *Igf2*, paternally expressed genes 1 and 3 (*Peg1,3*), Zinc Finger protein gene (*Zac1*), *Snrpn* and Long QT intronic transcript 1 (*Lit1*) gene (412).

Youngson et al. recently conducted a rat model study on the effects of paternal HFD for 13 weeks prior to mating on DNA methylation in paternal sperm and offspring tissues, with particular interest in identifying the genomic regions most vulnerable to paternal diet (413). They reported significantly higher global methylation levels and methylation of satellite repeats in paternal spermatozoa from the HFD group compared to the control (*p* < 0.05). Between the two groups, there were no significant differences in the satellite repeat methylation levels in the offspring’s liver, muscle and sperm, and in the methylation of either intracisternal-A particle long tandem repeats or LINE in the paternal spermatozoa or offspring tissues (413). There was no significant effect of paternal obesity on the methylation of imprinted regions *H19* ICR, *Peg3* DMR and *Snrpn* DMR (413).

Existing literature points to the potential effects of paternal diet, especially HFD, on the DNA methylation of the sperm epigenome. These studies raise a concern that imprinted genes may be more vulnerable to paternally-induced modulations of DNA methylation. Future studies exploring different nutrients within the paternal diet and in combination with altered maternal diet may shed more light on the interaction between maternal and paternal nutritional factors,
and provide more information on the specific mechanisms and possible long term health consequences of these influences.

2.3.3.3 Potential explanations for the inheritance of paternal environmental cues to the offspring

Although the results of the studies above indicate that paternal contribution to fertility and the health of the offspring should not be overlooked, the explanations behind the observed effects remain as theories. As mentioned in a previous section, histones are normally replaced by protamines in spermatozoa. However, this process is not perfect, and human spermatozoa can retain 15% of nucleosome- and histone-bound regions (379). These paternally conserved regions have been shown not to be random, but important in development, including promoters for signal factors, \textit{HOX} genes and imprinting regions (414). Structurally, histone-bound regions are more vulnerable to damage compared to those tightly bound to protamines because spermatozoa lose their cytoplasm during normal spermatogenesis, and the loss of scavenging enzymes within the cytoplasm results in reduced protection for histones from oxidation (415). Hence, the process of genetically transmitting pathologies to the offspring may begin with defective chromatin remodelling during spermiogenesis, the release of these vulnerable spermatozoa into seminiferous tubules with increased risk of DNA strand breaks, resulting in apoptosis or oxidative DNA damage.

In terms of the offspring’s inheritance of epigenetic changes through the spermatozoa, the aforementioned transition from histones to protamines depend on histone acetylation, which is regulated by histone deacetylases and acetylases. It has been shown that metabolic state can decrease the expression of histone deacetylase \textit{SIRT6}, resulting in increased histone acetylation and DNA damage in transitional spermatids and spermatozoa (416). Following fertilization, spermatozoon protamines are normally replaced by maternal histones. However, paternally bound histone regions are not replaced by the oocyte, so the modifications in these segments are inherited by the embryo (417). Moreover, the human spermatozoa contains around 1700 microRNAs, which are short, endogenous, single-stranded noncoding RNAs that can degrade gene transcripts (418). During fertilization, microRNAs can be transferred to the oocyte and cause phenotypic changes (419). It has been shown that microRNA injection into mouse embryo is associated with persistent pigmentation loss, cardiac hypertrophy, increased growth trajectories, in the following 2-3 generations (417). Therefore, one might be able to explain the
effects of paternal environmental factors on the sperm, onwards to the offspring through a complex interplay between genetic and epigenetic mechanisms.

A large body of evidence identifies the intrauterine period as the most susceptible time for the offspring to be influenced by maternal dietary, lifestyle and environmental cues. For the contribution of paternal factors to the offspring, Soubry et al. highlighted four potentially susceptible time windows (420). The first is during embryonic development, following implantation, when genome-wide active and passive demethylation occur. It has been shown that some genomic regions, including IAPs and LTRs, retain their methylation patterns during this period of erasure (421). In this manner, the protected paternal epigenetic marks can be transferred to the offspring. The second window of susceptibility is in the paternal prepuberty stage, when primordial germ cells differentiate into spermatogonia (420). It has been suggested that de novo methylation occurs at imprinted genes at this stage, as well as in the next stage – the third window of susceptibility - when spermatogonia mature into spermatocytes. Because de novo methylation is a highly dynamic process dependent on methyltransferase activity and the supply of methyl groups, paternal environmental factors may play an important role in this window (420). In fact, exposure to chemicals or pesticides (422), heavy metals (423), low dose ionizing radiation (424), chronic conditions (179), obesity (425) or increasing fatty acids (426) can stimulate the production of reactive oxidative species (ROS), which can alter sperm DNA methylation. The final susceptible window is the periconceptional zygote stage, during which some paternal histones resist being replaced by protamines, and are transferred to the oocyte (379). Such inherited epigenetic information within the paternal histones that are important for imprinting and early embryogenesis can influence the transcription and expression of specific genes in the offspring (420).

Although different theories exist to explain the observed effects of paternal environmental and nutritional factors on the sperm genome and the health of the offspring, it is important to investigate the mechanisms behind the results of these pioneering studies in order to shed more light onto a topic that had been untouched for a long time. With clearer explanations of the paternal genetic and epigenetic contribution to sperm and their offspring, further studies can be conducted to explore the importance of both maternal and paternal environmental cues on the offspring. In addition, studies up to date have focused on the effects of paternal obesity on the sperm. Future studies that focus on the various environmental exposures and nutritional factors are warranted to broaden the perspective on paternal influences.
2.3.3.4 The effect of environmental factors on paternal folate status

For many years, there has been an interest in environmental factors that modulate one’s folate status. In several countries, studies have shown that smoking is significantly associated with reduced folate and vitamin B<sub>12</sub> concentrations in the plasma and RBCs, and buccal mucosa folate levels (427–429). Wallock et al. conducted an observational study in the United States with healthy male smokers and non-smokers to examine the relationship between cigarette smoking and measures of plasma folate levels and semen quality (430). They reported that the percentage of folates that were non-methylTHFs (THF, 10-formylTHF, and 5,10-methyleneTHF) was significantly lower in the plasma of smokers compared to non-smokers (p < 0.05). However, their results demonstrated opposite effects of those previously published. Blood and seminal plasma folate concentrations were elevated in smokers in comparison to non-smokers, and blood plasma homocysteine concentrations were reduced in smokers (430).

Alcohol intake has also been linked to folate metabolism and can alter paternal folate levels. There is evidence that excessive alcohol intake in combination with low folate status promote cancer development (20). Generally, excessive alcohol intake has been shown to reduce folate bioavailability in a number of ways. Heavy drinkers (> 100 g alcohol/day) have been shown to have inadequate dietary folate intake (431), and chronic alcohol use has been shown to decrease intestinal and renal folate absorption (432,433). In fact, recent studies have explained malabsorption with ethanol intake through reduced expression of folate transporters, including PCFT, RFC and folate binding protein (FBP) in intestinal and renal membranes (434–436).

Wani et al. have also shown that chronic alcoholism in rats is associated with reduced hepatic folate uptake due to decreased expression of Pcft and Fbp in the hepatic basolateral membrane (437). The expression of these folate transporters may be regulated by epigenetic mechanisms. Wani et al. previously demonstrated that rats that received ethanol for 3 months had tissue-specific hypermethylation of these folate transporter genes (438). In addition, they reported hypermethylation and decreased expression of Fpgs, which encodes for folylpolyglutamate synthase that adds glutamate residues to monoglutamated folates (438).

The effects of alcohol on folate metabolism is predominantly through inhibiting MS, as is also suggested for the effects of cigarette smoking (20). Although the mechanism is not clearly elucidated, a number of studies have shown that ethanol administration inhibits MS activity (439,440). Recently, Wallock-Montelius et al. reported that chronic ethanol
consumption in micropigs increased testis folate concentrations and decreased testis MS activity (441). Before that, Halsted et al. utilized a micropig model to demonstrate that chronic and excessive alcohol ingestion was significantly associated with a 35% reduction in hepatic MS activity ($p < 0.001$) (440). Barak et al. and Halsted al. reported that, due to impaired MS activity from ethanol, there was subsequent decrease in downstream products, methionine and SAM, but an increase in the precursors, SAH and homocysteine (439,440).

Human studies have additionally shown two-fold greater plasma homocysteine levels ($p < 0.001$) and lower RBC folate concentrations ($p < 0.01$) in chronic alcoholics compared to the control (442). In 2005, Robinson et al. observed markedly high plasma homocysteine levels (> 15 μM) in both females and females with severe alcohol dependence (443). Alcohol-induced decrease in the SAM:SAH ratio may reflect onto DNA methylation. In fact, Choi et al. have shown in a rodent model that ethanol ingestion leads to a lower SAM:SAH ratio from SAH increase, resulting in global DNA hypomethylation in the colon (444). However, other studies have reported that, when folate availability is reduced and SAM:SAH ratio decreases, promoter methylation increases, leading to transcriptional gene silencing (445,446). These effects have have been attributed to compensatory heightened DNMT activity in times when low folate status would normally decrease methylation (445,446). However, Marutha Ravindran & Ticku have shown the opposite effect, where chronic ethanol treatment in mice led to promoter hypomethylation of a neurotransmitter gene (447).

The other aspect of the MS-inhibiting effect of alcohol is in an elevated conversion of 5,10-methyleneTHF to 5-methylTHF, resulting in its accumulation (20). In a rat model study, Hidiroglou et al. reported unchanged hepatic folate concentrations with 4 weeks of ethanol intake, however the proportion of 5-methylTHF in the folate pool increased by 45% while the other forms of folate decreased by the same percentage ($p < 0.01$) (448). Such accumulation of 5-methylTHF inhibits normal nucleotide biosynthesis, and has been shown by Halsted et al. to increase DNA strand breaks in the liver (440).

In summary, environmental factors such as smoking and alcohol have been shown to alter folate bioavailability and metabolism. There is a need for examining their implications on DNA methylation and synthesis pathways, as well as the role of paternal dietary factors on folate supply, metabolism and DNA methylation.
2.3.4 Paternal folate status and DNA methylation in the offspring

Although a few animal and human studies have shown that folate deficiency increases sperm DNA fragmentation and mutation frequency (449,450), Aarabi et al. was the first group to specifically examine the association between folic acid supplementation and changes to the sperm epigenome in humans (451). In a cohort of infertile men, they did not find significant changes to semen parameters or methylation of imprinted genes (H19, DKL1/GTL2, KCNQ1OT1, PLAG1, MEST, SNRPN) in sperm DNA with 6 months of folic acid supplementation. However, they did report significant global hypomethylation with supplementation, particularly in intergenic and low-density CpG regions. Additionally, methylation levels were markedly different among the MTHFR genotypes, where sperm from men without the MTHFR C677T polymorphism exhibited global hypomethylation only in intergenic regions. On the other hand, sperm from heterozygous men or homozygous for the MTHFR C677T polymorphism exhibited significant hypomethylation in promoters, exons, introns and intergenic regions ($p < 0.05$) (451). They reported that folic acid supplementation modulates sperm methylation patterns of genes involved in cancer and neurobehavioral disorders. Genes linked to gastrointestinal cancers were generally hypermethylated, and those involved in endocrine cancers were mainly hypomethylated.

Recently, a number of rodent model studies have studied the effects of paternal folate status on DNA methylation changes and the folate status of the offspring. Kim et al. investigated the effects of paternal folate deficiency (0 mg folic acid/kg) and supplementation (8 mg folic acid/kg) for 4 weeks on the global and gene-specific DNA methylation in the placenta and fetus on day 20 of gestation (452,453). Compared to the supplementation group, the folate deficient group had significantly lower placental weight ($p < 0.05$) and folate concentration of the placenta ($p < 0.01$) and fetal liver ($p < 0.05$), but greater placental $FReα$ expression ($p < 0.01$) (453). They additionally reported significantly lower global DNA methylation levels and downregulated Igf-2 expression in the fetal brain of the paternal folate deficient group compared to the supplemented group ($p < 0.0001$), although there was no significant difference in fetal brain folate content between the two groups (452). They reported significant correlations between paternal folate content in the liver and testes, and global DNA methylation and Igf-2 expression in the fetal brain ($p < 0.05$). Since $Fra$ is important for folate uptake, and Igf-2 in fetal growth, the two studies demonstrated that paternal folate deficiency may affect the expression of crucial genes involved in development. In 2013, Mejos et al. conducted a rat...
model study to examine the effects of both maternal and paternal folate intake on their offspring (454). Folic acid deficient (FD) and folic acid supplemented (FS) diets were given to randomly selected males and females, resulting in four groups – paternal deficient (PD), paternal supplemented (PS), maternal deficient (MD) and maternal supplemented (MS). Mating produced 4 groups of pups that were sacrificed at 21 days of age – PDxMD, PDxMS, PSxMD, PSxMS (454). Compared to the pups from the supplemented parents (PSxMS), the pups from the other groups had significantly reduced hepatic folate content \((p < 0.0001)\) and global DNA methylation \((p < 0.001)\). Moreover, hepatic folate content in the pup was significantly positively correlated with hepatic global DNA methylation \((p < 0.01)\). However, no differences in hepatic gene expression were detected for \(FR\alpha, IGF-2\) or \(IGF-1R\) among the groups.

Additionally, Lambrot et al. conducted an extensive mouse model study to examine the effects of paternal folate deficiency on the sperm epigenome and pregnancy outcomes (455). Male mice received either a folate sufficient (2 mg folic acid/kg) or deficient (0.3 mg folic acid/kg) diet beginning \textit{in utero} with the dams receiving either of these diets. These mice received postnatal diets with the same level of folate exposure as \textit{in utero}. In folate deficient males, they observed a delay in meiotic onset in the testis, and increased DNA damage in spermatocytes \((p < 0.05)\) (455). However, they did not detect significant effects of folate intake on the histological morphology (size or cell count) of the somatic cells, sperm count, or body weight, testis or epididymis weights. In order to assess the effects of paternal folate intake on reproductive outcomes, the male mice remained on their assigned diet for 2-4 months, at which point they were bred with virgin CD-1 female mice on a control diet. The folate deficient group had a significantly lower pregnancy rate and greater post-implantation loss compared to the folate sufficient group \((p < 0.05)\) (455). Fetuses sired by folate deficient males had a greater frequency of developmental abnormalities compared to those sired by folate sufficient males \((p < 0.05)\) (455). These malformations included craniofacial, limb and musculoskeletal defects.

In this study, folate deficiency additionally led to epigenetic changes. Sperm from folate deficient males had altered methylation at 57 genomic regions compared to the folate sufficient males (455). The differentially methylated genes included 51 implicated in development (of the central nervous system, kidney, reproductive development, spleen, digestive tract, muscle) and 47 genes associated with several cancers and chronic human diseases. There were no differences detected in the promoter DNA methylation of imprinted genes studied (455). In the placenta from foetuses sired by folate sufficient or deficient males, no differences in methylation levels
were detected (455). However, using gene expression array identified 380 genes that were differentially expressed between the two groups in placenta, and 21 of these genes were shown to regulate gene transduction and cell signalling. From these results, Lambrot et al. demonstrated the effects of paternal folate status on several histological and epigenetic sperm parameters, fertility and reproductive outcomes, in addition to the effects on placental epigenome and developmental abnormalities in the offspring. Specifically, they identified an extensive list of regions and genes in the sperm and placenta that were altered in methylation levels or expression by folate deficiency. The involvement of these genes in regulating growth and development, and in the risk of developing cancer and chronic diseases warrant further animal and human studies that will investigate the effects of paternal folate intake on the epigenome of the offspring.

Overall, although the interest in paternal contribution to the epigenome of the offspring is novel and evidence for the role of paternal folate status and the DNA methylation in the health of the offspring is limited, the first few studies in this new field of epigenetic research reveal that the epigenetic picture of the offspring does not solely depend on maternal conditions.
Chapter 3: Rationale, hypothesis and objectives

3.1 Rationale

Following mandatory folic acid fortification in 1998, intakes and blood levels of folate and folic acid have significantly increased in the North American population. Furthermore, ~40% of the North American population are known to take folic acid containing supplements for possible, but as yet unproven, benefits (4). Studies have shown that the major driving force behind the dramatic increase in the intakes and blood levels of folate and folic acid is folic acid supplementation with minor contributions from ready-to-eat cereals and fortified foods (456). Although folic acid fortification has been highly successful in achieving its primary objective, the protection against the development of NTDs, an emerging body of evidence has suggested potentially adverse health effects of high intakes and levels of folate and folic acid including cancer promotion and epigenetic alterations, in particular changes in DNA methylation (14). DNA methylation is inversely related to gene expression and plays a critical role in genomic stability (17). Because DNA methylation is programmed during embryogenesis, aberrant or dysregulation of DNA methylation during this critical window of development has been shown to contribute to the development of chronic diseases including cancer in the offspring later on in life (298,457,458). DNA methylation programming during embryogenesis can be modulated by maternal nutrition during pregnancy, and studies have previously demonstrated maternal folic acid supplementation alone or in conjunction with other dietary manipulations can significantly modify global and gene-specific DNA methylation in the offspring with functional consequences (5,6). Whether or not maternal folic acid supplementation can influence DNA methylation and disease susceptibility in the offspring is a highly important issue as the developing fetus has been exposed to high levels of folate and folic acid due to FA fortification and periconceptional FA supplementation (111).

The role of paternal nutrition in the health of the offspring has lagged behind that of maternal nutrition but studies have recently begun to consider paternal contribution to the development of the offspring. Currently, the potential role of paternal folate status in the DNA methylation of the offspring is largely unknown. However, it is well established that males are likely to be exposed to environmental factors leading to folate insufficiency, including smoking, alcohol consumption, and low intake of foods containing folate (41). Recently, it has been shown that paternal folate deficiency may affect reproductive functions including spermatogenesis and sperm quality, birth outcomes (7) and folate metabolism in the placenta.
Mejos and colleagues have demonstrated that paternal folate deficiency significantly decreases the hepatic folate content and global DNA methylation in their rat offspring (42). The reduction in the offspring’s hepatic folate content is of particular interest as folate transport across the placenta depends on maternal folate concentration during gestation (43). Given that these offspring were from the folate deficient father but folate sufficient dam, the expected outcome was that hepatic folate concentrations to be similar between pups from the folate sufficient and deficient fathers. However, the observed decrease in hepatic folate content in the offspring resulting from paternal folate deficiency suggests that the folate content in the sperm may contribute to tissue folate concentrations at fertilization, which is maintained during fetal development until birth.

Lambrot and colleagues have shown that paternal folate deficiency can significantly modulate DNA methylation in the sperm of a host of genes related to the offspring’s central nervous and reproductive system development, behavior and muscle development (7). In addition, with folate deficiency, the genes linked to the onset and progression of autoimmune diseases, neurological and psychological disorders and cancers were differentially methylated in the sperm (7). The functional outcomes of the folate deficiency-associated DNA methylation changes in the sperm were evident in the differential gene expression in the placenta of the offspring (7). These observations suggest that some specific epigenetic patterns in the paternal genome may be conserved throughout the period of epigenomic reprogramming in the preimplantation embryo (7) Mejos and colleagues (43) have demonstrated a positive correlation between hepatic folate contents and protein expression of FR-α, IGF-2 and IGF-1R, genes that play a major role in folate uptake in the placenta (9, 10) and regulating fetal growth and development (44, 45).

Although the paternal contribution of folate deficiency to DNA methylation of the offspring has been investigated, to our best knowledge, only one study has examined the effect of paternal folic acid supplementation at the time of conception on DNA methylation of the offspring. In addition to the contribution of high maternal folate intake and folic acid supplementation, paternal folate intake and folic acid supplementation may also significantly influence DNA methylation and health outcomes of the offspring. This conjecture needs to be tested and confirmed in appropriate preclinical models and human studies. Given these considerations, my thesis focused on the effect of paternal folic acid supplementation at the time of mating on tissues folate concentrations and DNA methylation of the newborn pups in an well-established mouse model of folate manipulation.
3.2 Hypothesis

The paucity of studies examining the effect of paternal folate status on tissue folate concentrations and DNA methylation makes it difficult to hypothesize *a priori* the direction of the effect of paternal FA supplementation at the time of conception on the liver folate concentrations and gene-specific DNA methylation in the offspring. Although Aarabi et al. observed a loss in DNA methylation with folic acid supplementation (451), based on the previous observations concerning the effect of paternal folate deficiency on tissue folate concentrations and DNA methylation in the offspring, my *a priori* hypothesis is that paternal folic acid supplementation would be associated with increased folate concentration and DNA methylation of the selected genes in the liver of the offspring. Given the gene and site-specificity of folate status on DNA methylation, the effect of paternal FA supplementation on DNA methylation may be gene-specific (452,454,455).

3.3 General objectives

The main objective of this thesis was to determine the effects of paternal folic acid supplementation at the time of conception on plasma and tissue folate concentrations and gene-specific DNA methylation of the offspring in a mouse model. A secondary objective of this thesis was to examine the effects of paternal folic acid supplementation at the time of conception on breeding and birth outcomes.
Chapter 4: The effect of paternal folic acid supplementation on folate concentrations and DNA methylation of the offspring in a mouse model

4.1 Abstract

Background: DNA methylation is an important epigenetic determinant of gene expression, DNA integrity, and chromatin modifications, aberrancies of which are mechanistically related to the development of chronic diseases. DNA methylation is programmed during embryogenesis and is highly susceptible to intrauterine environmental modifiers including maternal diet. Folate plays a critical role in one-carbon transfer reactions involved in DNA methylation. Maternal folic acid supplementation has been shown to significantly modify global and gene-specific DNA methylation in the offspring with consequent functional ramifications. However, whether or not paternal folate status plays a role in DNA methylation programming in the developing fetus has not been well established. We investigated the effect of paternal folic acid supplementation on DNA methylation in the offspring.

Methods: Weanling male C57BL/6 mice (n=20) were randomized to a diet containing 2 (control) or 20 mg folic acid/kg diet. Weanling female mates (n=20) received the same control diet containing 2 mg folic acid/kg diet. After 3-4 weeks of dietary intervention, mating and breeding began. Male mice were maintained on their respective folic acid diet during mating, while female mates were kept on the control diet. All pups were sacrificed at birth. At necropsy, plasma was collected and liver, kidney, colon were harvested. Plasma and tissue folate concentrations were determined by the standard microbiologic assay. Gene-specific DNA methylation of the Ppar-α, Ppar-γ, Gr and Igf-2 were determined in the tissues by bisulfite conversion followed by pyrosequencing.

Results: There was no significant effect of paternal folic acid supplementation on the litter size, but paternal folic acid supplementation significantly decreased the number of days to pregnancy ($p < 0.001$). The plasma folate concentrations of the sires in the supplemented group were significantly higher than those in the control group ($p < 0.0001$), and there were no significant differences in plasma folate concentrations of the dams in the two paternal folic acid groups. There were no significant differences in the pups’ tissue folate concentrations between the two groups. Paternal folic acid supplementation did not significantly alter the DNA methylation of the Ppar-α, Ppar-γ, and Igf-2 genes but there was a significant sex effect on the DNA
methylation of the Igf-2 gene ($p < 0.05$). Paternal folic acid supplementation significantly increased DNA methylation of the Gr gene ($p < 0.05$).

**Conclusions:** Paternal folic acid supplementation may improve breeding efficacy. Although paternal folic acid supplementation had no significant effect on plasma and tissue folate concentrations of the newborn pups, it may affect DNA methylation of several genes involved in the growth and development of the offspring.

**4.2 Introduction**

Due to frequent unplanned pregnancies and the neural tube closing early in pregnancy, some countries, including Canada, have implemented mandatory food fortification with folic acid since 1998 to reduce the risk of NTDs (2). In Canada, folic acid containing supplements are used by up to 40% of the population for possible, but as yet unproven, health benefits and periconceptional folic acid supplementation is recommended to women of reproductive age to reduce the risk of NTDs (4). As a result, intakes and blood levels of folate and folic acid in Canada pregnancy women have significantly increased (5) with consequent drastically increased intrauterine exposure of the developing fetus to folate and folic acid. Although periconceptional folic acid supplementation and folic acid fortification have significantly decreased the NTD rates in Canada (3), concerns have been raised over whether or not the exposure of the developing fetus to high folate and folic acid may have adverse health effects on the offspring later in life.

Folate mediates the transfer of one-carbon moieties involved in nucleotide biosynthesis and hence, plays an important role during fetal growth and development (1). Furthermore, in the form of 5-methylTHF, folate mediates the transfer of one-carbon group in remethylation of Hcy to methionine and subsequently, SAM, the universal methyl group donor in most biological methylation reactions including DNA methylation. DNA methylation is an important epigenetic inverse determinant in gene expression and critically contributes to maintenance of genomic stability, aberrancies of which are pathogenically related to the development of chronic diseases including cancer (23). During embryogenesis, DNA programming takes place and a new pattern of DNA methylation is established and maintained through early life and adulthood (459). Thus, DNA methylation during embryogenesis is highly susceptible to environmental modifiers including maternal dietary factors including folate (1). A number of studies have demonstrated that maternal folic acid supplementation provided *in utero* significantly changes global and
gene-specific DNA methylation in the offspring and modifies cancer susceptibility in adulthood in rat models (10,12). Given the drastically increased exposure of the developing fetus to folate and folic acid in the North American population, whether or not this will influence DNA methylation of the offspring and thus, cancer susceptibility in adulthood is a significant public health concern.

Although the role of paternal nutrition, including folate status, on DNA methylation and modifications of disease susceptibility in the offspring has not been well characterized, studies are beginning to emerge. Folate deficiency in males is known to affect reproductive functions such as spermatogenesis and sperm quality (460), which is particularly important as men are likely exposed to environmental factors that may increase the risk of folate deficiency, including smoking, alcohol consumption, and low intake of fruits and vegetables (461). A recent study has demonstrated that paternal folate status has a role in the folate metabolism in the liver and the placenta (453,454), and the decreased expression of IGF-2 and global DNA methylation in the fetal brain (452). Lambrot and colleagues have also recently shown that paternal folate deficiency is associated with changes in the sperm epigenome and with negative birth outcomes (455).

A recent Canadian study using the Canadian Health Measures Survey has reported that folate deficiency in the Canadian population is exceedingly rare and approximately 40% of Canadian men 20 to 59 years of age had RBC folate concentrations in the range considered to be high (greater than the 97th percentile of NHANES (1998-2004) data) (2). This suggests that paternal folate status at the time of conception in the Canadian population is likely high. At present, although one recent reported significant effects of folic acid supplementation on sperm DNA methylation, no studies have examined the effect of paternal folic acid supplementation at the time of conception on DNA methylation in the offspring. Therefore, we investigated the effects of paternal folic acid supplementation on plasma and tissue folate concentrations in the offspring and determined whether paternal plasma folate concentrations are correlated with plasma and tissue folate concentrations in the offspring. Furthermore, we determined whether paternal folic acid supplementation affects gene-specific DNA methylation of the Ppar-α, Ppar-γ, Gr and Igf-2 genes, which are involved in fetal development and growth and assessed for any association between paternal plasma folate concentrations and DNA methylation of these genes in the offspring. We also examined the impact of paternal folic acid supplementation on breeding patterns and the rate of successful pregnancy.
4.3 Materials and Methods

4.3.1 Animals and dietary intervention

This study was conducted in strict accordance with the Regulations of the Animals for Research Act in Ontario and the Guidelines of the Canadian Council on Animal Care. The study protocol was approved by the Animal Care Committee of St. Michael’s Hospital (Toronto, ON). Post-weaning (3 weeks of age) male and female C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, QC). Upon arrival, male mice were randomized to receive an amino-acid defined diet (Dyets, Bethlehem, PA) containing either 2 (control; basal daily requirement) or 20 mg folic acid/kg diet for 3-4 weeks prior to and during mating (Figure 4.1).

![Study design diagram]

**Figure 4.1: Study design.**

All female mice received the same control diet containing 2 mg folic acid/kg diet prior to and during mating and throughout pregnancy. Water was provided daily while diets were replaced twice weekly. Water and diets were provided *ad libitum*. Food intake was monitored weekly to ensure similar food consumption between the two diet groups. Furthermore, animal health was monitored daily and the body weight recorded weekly.

4.3.2 Breeding strategy

After 3-4 weeks of dietary intervention, when the mice were about 6 to 8 weeks of age, which marks their sexual maturity, breeding was initiated. Each male mouse was placed in a cage to mate with one female mouse (no one male mouse had more than one female mating
partner, and female mice had only one male mating partner) under light/dark cycles over 12 hours. To prevent the male mouse in the folic acid-supplemented group (20 mg folic acid/kg diet) from consuming the control diet (2 mg folic acid/kg diet) of the female mating partner, food was removed from the female cage during mating. After mating overnight, the male mouse was returned to its own cage and consumed its own diet (either 2 or 20 mg folic acid/kg diet). Pregnancy was confirmed by monitoring weight changes over the course of one week, and identifying the presence of a vaginal plug using a metal probe. Pregnancy female mice consumed the control diet throughout pregnancy.

4.3.3 Experimental diets

Amino acid-defined diets containing different amounts of folic acid constitute a standard method of providing supplemental dietary folic acid in rodents in a predictable manner (462) and have been extensively used in previous rodent studies including those that examined the effects of maternal folic acid supplementation on DNA methylation and cancer risk in the offspring (463,464). The detailed compositions of the diets are found in Table 4.1 and Table 4.2. The control diet containing 2 mg folic acid/kg diet is generally accepted as the basal dietary requirement for rodents (465). This diet contains approximately 4000 kcal/kg diet, which translates to 0.5-1 mg of folic acid in 2000 kcal. This level of folic acid expressed relative to caloric content very closely approximates the RDA in humans consuming a daily average of 2000 kcal (27) and thus, was selected to reflect the RDA for folate in humans. The 20 mg folic acid/kg diet represents folic acid supplementation at 10x the basal dietary requirement and was selected to approximate the intake of 4-5 mg folic acid in humans. In the Canadian population, this level of supplementation is used for some patients with cancer and inflammatory conditions receiving anti-folate-based treatment to ameliorate the adverse effects of anti-folates (466). Mice exhibit more efficient folic acid biotransformation compared humans due to a comparatively high DHFR activity relative to humans (467). Consequently, mice would have to ingest a much greater than pro-rata amount of folic acid in order to elicit the same circulating plasma concentrations of folic acid as humans in order to assess the impact of systemic exposure of folic acid (63,467). Therefore, the selected 10x supplemental level of dietary folic acid in mice in the present study likely achieved much lower plasma concentrations of folic acid than would have been achieved by the equivalent supplemental levels of folic acid in humans. Thus, the 10x supplemental level of folic acid was chosen to account for this reported differences in folic acid biotransformation between mice and humans and to maximize the change of observing any
The effect of paternal folic acid supplementation on DNA methylation in the offspring as proof-of-principle.

Table 4.1: Nutrient compositions of experimental L-amino acid defined diets

<table>
<thead>
<tr>
<th>Nutrient (g/kg of diet)</th>
<th>2 mg FA/kg</th>
<th>20 mg FA/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cat # 517774</td>
<td>Cat # 517801</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Arginine free base</td>
<td>11.2</td>
<td>11.2</td>
</tr>
<tr>
<td>L-Asparagine.H2O</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>23.3</td>
<td>23.3</td>
</tr>
<tr>
<td>L-Histidine free base</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>11.1</td>
<td>11.1</td>
</tr>
<tr>
<td>L-Lysine HCl</td>
<td>14.4</td>
<td>14.4</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>11.6</td>
<td>11.6</td>
</tr>
<tr>
<td>L-Proline</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Serine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>1.74</td>
<td>1.74</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>L-Valine</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td><strong>Total L-amino acid</strong></td>
<td><strong>171.44</strong></td>
<td><strong>171.44</strong></td>
</tr>
<tr>
<td>Dextrin</td>
<td>407</td>
<td>407</td>
</tr>
<tr>
<td>Sucrose</td>
<td>194.6</td>
<td>191</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn Oil (w/0.015% BHT)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Salt Mix #210006</td>
<td>57.96</td>
<td>57.96</td>
</tr>
<tr>
<td>Vitamin Mix #317756 (no Folate)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td>Folic Acid/sucrose premix 5mg/g</td>
<td>0.4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1000</strong></td>
<td><strong>1000</strong></td>
</tr>
</tbody>
</table>
Table 4.2: Salt mix and vitamin mix compositions of experimental L-amino acid defined diets

<table>
<thead>
<tr>
<th>Salt Mix # 210006</th>
<th>Ingredients (g/kg of diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium carbonate</td>
<td>14.6000</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
<td>0.17000</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>12.37000</td>
</tr>
<tr>
<td>Potassium phosphate, dibasic</td>
<td>17.16000</td>
</tr>
<tr>
<td>Magnesium sulfate, anhydrous</td>
<td>2.45000</td>
</tr>
<tr>
<td>Manganese sulfate, monohydrate</td>
<td>0.18000</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.62000</td>
</tr>
<tr>
<td>Zinc carbonate</td>
<td>0.05400</td>
</tr>
<tr>
<td>Cupric carbonate</td>
<td>0.05400</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.00058</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>0.00058</td>
</tr>
<tr>
<td>Chromium potassium sulfate</td>
<td>0.01900</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>0.00230</td>
</tr>
<tr>
<td>Molybdic acid, ammonium salt</td>
<td>0.00120</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.27534</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin Mix # 317756</th>
<th>Ingredients (g/kg of diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamin HCl</td>
<td>0.006</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.006</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.007</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.030</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>0.016</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>0.00005</td>
</tr>
<tr>
<td>Vitamin A palmitate (500 000 IU/g)</td>
<td>0.008</td>
</tr>
<tr>
<td>Vitamin D3 (400 000 IU/g)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Vitamin E acetate (500 IU/g)</td>
<td>0.100</td>
</tr>
<tr>
<td>Menadione sodium bisulfate</td>
<td>0.00080</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.00002</td>
</tr>
<tr>
<td>Sucrose</td>
<td>9.82363</td>
</tr>
</tbody>
</table>

4.3.4 Sample collection

At the time of birth, the sire, dam and pups were killed. The sires and dams were killed by 5% isoflurane inhalation followed by cardiac puncture and cervical dislocation. Their blood was collected by cardiac puncture in heparinized vacutainer and placed on ice and protected from light immediately. The pups were killed by decapitation using surgical scissors, and their blood was collected using glass capillary tubes (VWR International, City, State or Province) with ammonium heparin as an anticoagulant to keep the blood from clotting. Plasma was collected by centrifuging the blood at 6000 x g for 15 minutes at room temperature. The plasma
was treated with 10% sodium ascorbate and stored at -80°C until plasma folate analysis. The liver, colon and kidneys from each mouse was harvested at necropsy, immediately snap frozen in liquid nitrogen, and stored at -80°C for tissue folate assay and DNA extraction.

4.3.5 Determination of plasma and hepatic folate concentrations

Plasma and tissue folate concentrations were determined by the standard microbiological microtitre plate assay with *Lactobacillus rhamnosus* (also known as *Lactobacillus casei*), which is considered to be the “gold standard” in the determination of folate concentrations (58, 59). Because the growth of *L. rhamnosus* is solely dependent on external sources of folate, the bacterial growth is proportional to the folate content in medium (58). Thus, the growth of the bacteria in culture, measured by the optical density of the media, reflects the folate concentrations of a sample.

**Folic acid standard preparation:** 10 mg of folic acid was dissolved in 10 mL of double-distilled water (ddH₂O) with 5 µL of 10M NaOH to attain a final concentration of 1 mg folic acid/mL. The solution was adjusted to a pH of 7-8 using HCl, and the pH value was verified using spectrophotometry at 282 nm. Thereafter, the solution was diluted to 50 µg/mL using methanol, and aliquots were stored at -80°C for future use.

**Lactobacillus rhamnosus stock preparation:** *L. rhamnosus* ATCC 7649 stock was incubated with *Lactobacillus* MRS Broth (Difco™, BD Biosciences) (200 µL in 200 mL) for 18 hours at 37°C. Under aseptic conditions, the cells were centrifuged and the supernatant was decanted. The cell pellet was resuspended in 180 mL of *Lactobacillus* MRS Broth and 20 mL of autoclaved 100% cold glycerol. The solution of stock bacteria was thoroughly mixed and aliquots were stored at -80°C for future use.

**Chicken pancreas conjugase preparation:** Chicken pancreas acetone powder (Difco™, BD Biosciences) was dissolved in sterile filtered 0.1M KPO₄ buffer (1.05 g KH₂PO₄, 0.4 g K₂HPO₄, 0.1 g C₆H₇NaO₆ and 100 mL ddH₂O). The solution was then incubated under a blanket of toluene for 6 hours at 37°C, after which the toluene was removed. The solution was centrifuged at 10 000 x g for 15 minutes, and the supernatant was collected. To it, an equal volume of tricalcium phosphate (BioRad Gel HTP was rehydrated: 1 part HTP to 6 parts of 0.1M KPO₄ buffer per 10 g HTP) was added. The solution was mixed at 4°C for 30 minutes and centrifuged at 10 000 x g for 30 minutes at 4°C. Following the collection and cooling of the supernatant, an
equal volume of 95% ethanol was added to it, mixed and stored overnight at -20°C. The next day, the solution was centrifuged at 10 000 x g for 30 minutes, the supernatant was collected and resuspended in 50 mL of cold 1.0M KPO₄ buffer. To the solution was added with 10 g of Dowex-1 (BioRad AG1-X8) and mixed for 1 hour at 4°C. Thereafter, the solution was filtered using Whatman #1 filter paper at 4°C, and aliquots stored at -80°C for future use.

**Tissue folate extraction:** The liver, kidney and colon were used for the assessment of hepatic folate concentrations. The samples were weighed and 2.5 mL of extraction buffer (0.1M C₆H₇NaO₆, 0.1M Bis-Tris, and 5mM β-mercaptoethanol) per 0.25 g of tissue was added to each sample. They were boiled in water for 15-20 minutes and cooled on ice for 15-20 minutes. The mixture was then centrifuged at 5 000 rpm at room temperature for 1 minute and homogenized. Thereafter, the samples were centrifuged at 5 000 rpm for 20 minutes at 4°C, and the supernatant was collected and stored at -80°C for future use. The tissue folate extract was incubated with chicken pancreas conjugase and 0.1M KPO₄ buffer at a 15:1:4 ratio for 2 hours at 37°C. The samples were further diluted by a factor of 9 with 0.1M KPO₄ buffer and stored at -80°C.

**Determination of plasma and tissue folate concentrations:** Three µL of *L. rhamnosus* was inoculated into 3 mL of *Lactobacillus* MRS Broth, and the bacterial culture was incubated in a shaker at 250 rpm and 37°C for 16-18 hours overnight. The overnight culture (500 µL) was added to 2.5 mL of *Lactobacillus* MRS Broth and incubated for another 6-6.5 hours under the same conditions as the previous day. Using a spectrophotometer at a wavelength of 650 nm, the optical density was measured and bacterial growth was confirmed.

Using a sterile and clear 96-well flat bottom plate, 150 µL of fresh KPO₄ buffer was added to each well. To the starting wells, 150 µL of the folic acid standard (diluted to 2 ng/mL) was added in duplicates and serially diluted by two folds in order to produce an eight point standard curve. For each plasma sample, the folate content was determined in duplicate by adding 2.5 µL or 1.25 µL of sample from the pups from fathers that received 2 mg folic acid/kg or 20 mg folic acid/kg in their diet, respectively, into designated starting wells. The volume was then adjusted to 300 µL by adding either 147.5 µL (for the pups from fathers that received 2 mg folic acid/kg) or 148.75 µL (for the pups from fathers that received 20 mg folic acid/kg) of the KPO₄ buffer. To assess tissue folate concentrations, 5 µL of the sample was added into designated starting
wells and the volume was adjusted to 300 µL with the addition of 145 µL of KPO₄ buffer. Both the plasma and tissue samples were then 2-fold serially diluted across three additional wells, providing four measurements.

For the optical density measurement, the *L. rhamnosus* culture was thoroughly washed five times and resuspended in 3 mL of freshly-prepared folic acid casei media (a mixture of 9.4 g Folic Acid Casei Medium [Difco™, BD Biosciences], 0.05 g C₆H₇NaO₆ and 100 mL ddH₂O that was boiled and sterile-filtered) in order to remove any remaining folates from the extremely folate-rich *Lactobacillus* MRS Broth. The resulting bacterial culture was step-wise diluted by a cumulative factor of 1000 X, and 150 µL of the culture was added to each well of the 96-well plate. The plate was then sealed with Mylar and incubated for 16-18 hours at 37°C, after which the optical density of each well was measured using a spectrophotometer at a wavelength of 650 nm. Finally, the sample folate concentrations were interpolated from the generated standard curve of folate concentrations using SoftMax→ Pro V5.4.1 software (Molecular Devices, CA).

4.3.6 DNA extraction

**Genomic DNA isolation:** DNA from the liver was extracted by the DNeasy→ Blood & Tissue Kit (Qiagen, City, State/Province) according to the manufacturer’s instructions with modifications. Half of the liver harvested during sample collection was first added to 360 µL of Buffer ATL, a tissue lysis buffer that is used to purify nucleic acids. After homogenization, the mixture was left to settle into liquid before transferring into a 1.5 mL microcentrifuge tube. Forty µL of proteinase K was added, and the contents of the tube were thoroughly mixed and incubated in Eppendor Thermomixer Comfort (Eppendorf) overnight at 56°C with the shaking mode on. To this mixture, 8 µL of Ribonuclease A (RNase A) (Qiagen) was added for RNA digestion, mixed and incubated for 2 minutes at room temperature. After mixing by vortex for 15 seconds, 400 µL of premixed buffer (200 µL AL [Qiagen] and 200 µL 96-100% ethanol) was added to the sample and thoroughly mixed again. The mixture was transferred into a DNeasy Mini spin column placed in a 2 mL collection tube (Qiagen) and centrifuged at 6000 x g for a minute. The flow-through and collection tube were then discarded. The spin column was placed in a new 2 mL collection tube, into which 500 µL of Buffer AW1 (Qiagen) was added, and centrifuged at 6000 x g for one minute. The flow-through and collection tube were discarded. The spin column was placed in a new 2 mL collection tube, into which 500 µL of Buffer AW2 (Qiagen) was added, and centrifuged at 20 000 x g. The flow-through and collection tube were
discarded, and the spin column was placed in a new 1.5 mL microcentrifuge tube. The DNA was eluted by adding 200 µL Buffer AE to the center of the spin column membrane, incubating for 1 minute at room temperature, and centrifuging for 1 minute at 6000 x g. The spin column was placed in another 1.5 mL microcentrifuge tube and eluted again with the same amount of Buffer AE. The previously eluted DNA was combined with this DNA in order to increase the DNA yield.

**DNA precipitation:** To the DNA samples, 40 µL of 10% sodium acetate and 800 µL of ice-cold 100% ethanol were added. After the mixture was gently mixed, it was stored at -20°C overnight. The sample was centrifuged at 13.2 rpm at 4°C for 30 minutes, after which the liquid was discarded, making sure not to lose the pellet of DNA in the bottom of the tube. One mL of ice-cold 70% ethanol was added to the tube and the liquid was discarded once again, making sure not to lose the pellet. Finally, the DNA was resuspended with 20 µL of Buffer AE. The DNA quantity was measured and the DNA purity was confirmed using a NanoDrop 2000 (Thermo Scientific) instrument for spectrophotometric measurement of nucleic acid concentrations, pertinent absorbance values and calculation of absorbance ratios. The size of DNA estimated by agarose-gel electrophoresis was more than 20 kb in all instances. The final preparations had a ratio of A260 to A280 between 1.8 and 2.0 and were free of RNA and protein contaminations. The concentration of each DNA sample was determined as the mean of three independent spectrophotometric readings. The DNA was stored at -20°C for DNA methylation assay.

4.3.7 **Sex determination:**

The anatomic sex determination of a newborn pup using the anogenital distance (AGD) is very difficult to impossible and inaccurate (60). Therefore, the extracted DNA from the liver samples was used to determine sex of each pup by a polymerase chain reaction (PCR)-based assay at the Genetic Analysis Facility, the Centre for Applied Genomics, the Hospital for Sick Children (Toronto, Ontario). This assay is based on that established by Clapcote and colleagues (61) with minor modifications. In brief, this assay utilizes one pair of primers in a single reaction tube to simultaneously amplify DNA fragments from both the X- and Y-chromosomes (61). The sequences used for designing the primers for this assay are from the X-chromosome-specific gene Jarid1c and the Y-chromosome-specific gene Jarid1d, and the specific pair of primers (forward: 5’-CTGAAGCTTTTGGCTTTGAG-3’, reverse: 5’-CCACTGCCAAATTCTTTTG-3’) simultaneously amplifies DNA fragments of 331 bp from
*Jarid1c* and 302 bp from *Jarid1d* (61). The reactions were carried out in 8-well tube strips (Becton Dickinson, Mississauga, ON, Canada), with each PCR mixture consisting of nuclease-free water, 10x reaction buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton® X-100), 1.6 mM MgCl₂, 0.2 mM of dATP, dTTP, dCTP and dGTP, 0.5 µM of each primer, 1.5 U *Taq* DNA polymerase and 100 ng genomic DNA (61). Following thermal cycling, each PCR product was mixed with 5 µL 6X loading dye solution (Fermentas Life Sciences, Burlington, ON, Canada) and loaded onto a 2% agarose gel next to a DNA ladder and electrophoresed (61). Sex determination was made on UV light transillumination of the gel after staining with ethidium bromide: single bands at 302 bp and 331 bp for male and female, respectively (Figure 4.2).

**Figure 4.2:** PCR products obtained by simultaneous amplification of X- and Y-chromosome-specific genes. Lanes 1, 2, 6, female pups; 3-5, male pups (C57BL/6). The sizes of DNA ladder fragments and PCR products are indicated.
4.3.8 Gene-specific DNA methylation determination by bisulfite conversion followed by pyrosequencing

The DNA methylation status of specific CpG sites of the \textit{Ppar-\textalpha, Ppar-\textgamma, Gr and Igf-2DMR2} genes in the liver DNA was determined by an assay utilizing bisulfite conversion and pyrosequencing as described previously (468) at the Hospital for Sick Children (Toronto, Ontario, Canada). This assay utilizes the Imprint DNA Modification Kit (Sigma, Cat\# MOD50) for bisulfite conversion and the PyroMark→Q24 system (Qiagen, Cat\# 9001514) for pyrosequencing, according to the manufacturer’s instructions.

Figure 4.3: Targeted CpG sites for gene-specific methylation in the liver of the pups at birth. 3 CpG sites within the first exon for \textit{Ppar-\textalpha} [A]. 5 CpG sites within the first exon for \textit{Ppar-\textgamma} [B]. 5 CpG sites within the third exon for \textit{Igf-2} [C]. 8 CpG sites within the second exon for \textit{Gr} [D].

The four genes were chosen because they play important roles in the growth and development of the developing fetus, and because they have been previously shown to be epigenetically regulated. \textit{Ppar-\textalpha} is responsible for activating genes involved in fatty acid oxidation and increasing the expression and activities of enzymes and transport proteins that facilitate and intracellularly deliver fatty acids (469). \textit{Ppar-\textgamma} activates genes involved in adipogenesis, increases the expression of genes involved in fat storage (lipoprotein lipase), fatty acid synthesis (acyl-CoA synthase), and glucose transport (GLUT4) (469). Hence, both \textit{Ppar-\textalpha} and \textit{Ppar-\textgamma} encode for transcription factors that regulate expression of important genes involved in lipid homeostasis and metabolism, and alterations to their expression from maternal protein
diet restriction has been associated with cardiovascular and metabolic changes to the offspring (226). In addition, high maternal multivitamin intakes (470), caloric restriction (471) and folic acid supplementation have all been shown to modulate \( Ppar \) expression in the liver, lungs, placenta and brain. The CpG sites assessed in the present study were selected using the UCSC Genome Browser (version 3) and reports from Carone et al. about the association between paternal protein restriction and altered promoter CpG methylation at \( Ppar-\alpha \) with associated elevation in hepatic gene expression (16). We also selected the CpG sites for \( Ppar-\gamma \) methylation based on reports from Fujiki et al. that showed decreased \( Ppar-\gamma \) expression from DNA methylation at its promoter region in adipose tissue of a diabetic mouse model (472).

\( Gr \), also known as \( Nr3c1 \) (nuclear receptor subfamily 3, group C, member 1), encodes for glucocorticoid receptor, which glucocorticoids bind to and activate. The glucocorticoid receptor is then a regulator a transcription for several genes necessary in important biological pathways, including inflammation and the metabolism of glucose, fat and bone (473). Previously, low maternal care, in the form of licking and grooming, has been shown to increase \( Gr \) promoter DNA methylation in the hippocampus (474). Furthermore, in a mouse model, paternal exposure to synthetic glucocorticoids has also been shown to decrease methylation in regulatory regions of \( Gr \) and alter its expression in the postnatal mouse kidney, suggesting that there is a role of stress-induced paternal effects on the epigenetics of the offspring (475). We selected the specific CpG sites chosen for the present study based on published data demonstrating the association between paternal nutritional factors and \( Gr \) promoter DNA methylation, and we also collaborated with Dr. Rosanna Wecksberg’s group to utilize the aforementioned Genome Browser in order to select >100 bp regions without single nucleotide polymorphisms or repeat sequences.

Finally, \( Igf-2 \) is a paternally imprinted gene that encodes for a growth factor specific to fetal development and is an indicator of postnatal growth and metabolism (476). Previous studies have shown that epigenetic changes in this gene are associated with a number of diseases, including Wilms tumour, Beckwith-Wiedemann syndrome, rhabdomyosarcoma and Silver-Russell syndrome (477). During gestation, its monoallelic expression from the paternal allele is conserved in most rodent tissues (478). Postnatally, \( Igf-2 \) is expressed in serum, plasma, liver, kidney, and intestine (479). For the present study, the gene was selected because the IGF-2 protein is primarily produced in the liver (480), and the chosen CpG sites are located in the DMR2, and DNA methylation of these regions have been shown to dictate the expression of the
imprinted gene (481,482). Dejeux et al. reported that hypermethylation of Igf-2DMR2 is associated with loss-of-imprinting and overexpression of the gene at the mRNA and protein levels (482). In the fetal brain, paternal folate deficiency has been shown to decrease Igf-2 DNA methylation and protein expression of IGF-2 (452). However, Mejos et al. used a rat model to demonstrate that no significant differences were found in IGF-2 protein expression among offspring born to sires and dams that were either folate-supplemented or folate-deficient (454).

Bisulfite sequencing is highly qualitative and quantitative in identifying 5-mC at a single base-pair resolution. Bisulfite treatment in the Imprint DNA Modification Kit converts unmethylated cytosines to uracil, which is amplified as thymine, but 5-mC is resistant to the change. During amplification, 5-mC is recognized as cytosine, and primer sequences are used to identify methylation status in the loci of interest. In brief, genomic DNA extracted from the liver was normalized to a concentration of 50-75 ng/µL, and 10 µL of DNA was added to a mixed solution of 1.1 mL of DNA Modification Solution and 1 vial of DNA Modification Powder. The tube was incubated at 99 °C for 6 minutes, then at 65 °C for 90 minutes. The modified DNA was cleaned with Balance/Ethanol Wash Solution elution, and ready for pyrosequencing.

The HotStarTaq Master Mix Kit (Qiagen) was utilized to amplify the DNA to be analyzed. The components of the PCR mixture were 25 µL of the HotStarTaq Master Mix, which included DNA Polymerase, a PCR Buffer containing 1.5 mM of MgCl₂, and 200 µM of each dNTP), 5 µL of primer mix with 0.2 µM of each primer in Table 4.3, RNase-free water and template DNA.

The PCR tubes were placed in the thermal cycler, and PCR cycling began with the initial heat activation step at 95 °C for 15 minutes. The 3-step cycling of denaturation, annealing and extension was carried out at 94 °C for 0.5-1 minute, 50-68 °C for 0.5-1 minute and 72 °C for 1 minute, respectively. Following 25-35 cycles, the final extension at 72 °C for 10 minutes completed the DNA amplification process. The primers were labelled with biotin, and the final PCR product was purified by binding them to Streptavidin Sepharose beads (Streptavidin Sepharose High Performance, GE Healthcare). The beads with PCR product were purified with the PyroMark Q24 Vacuum Workstation (Pyrosequencing Inc., Westborough, MA, USA), and the samples were annealed to sequencing primers at 80 °C for 5 minutes.

The PyroMark Q24 Instrument runs the pyrosequencing assay. As each dNTP is added to the reaction, if it is complementary to the base of the template strand (the purified PCR
product), the incorporation is accompanied by pyrophosphate (PPi) release. The quantity of release is equimolar to that of the incorporated nucleotide. ATP sulfurylase converts PPi to ATP, and luciferase converts luciferin to oxyluciferin. This reaction produces visible light in proportion to the amount of ATP, and the detected light is observed as a peak in the Pyrogram.

The height of each peak in the Pyrogram is proportional to the number of incorporated nucleotides. As this sequential addition of dNTPs continues, the complementary DNA strand is elongated, and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace. The level of methylation (%5mC) for each DNA locus was reported as a percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines.

**Table 4.3 Primer sequences used for RT-PCR and pyrosequencing**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>PCR Product Size</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ppar α</td>
<td>Forward</td>
<td>TGTTAGTAGTTGTTTTGGGGTTG</td>
<td>86bp</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCCTCCAAAAAAACAACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>TGGGGGTTGGAGGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ppar γ</td>
<td>Forward</td>
<td>GGGTTGAGAGAGTTATAAAGAGAA</td>
<td>83bp</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AACCCCTCACTCTATCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>GGAGAAGTTATATAAGAGAAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Igf-2</td>
<td>Forward</td>
<td>GGGTGGGGGGGTGTTTTTATGG</td>
<td>265bp</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCACCCCTAATTCCTTCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>GAATTTTATGGTAGGTTTTAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr</td>
<td>Forward</td>
<td>AGAGTTTAGAGGGGGGTAGATGT</td>
<td>222bp</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTAACCCTCTACTCTACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequencing</td>
<td>GAAGTGTTATTATTAGTAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.9 **Statistical analysis**

**Sample size calculation**

Since folic acid study results are variable from experimenter to experimenter, there is not much information for what value of standard deviation is “meaningful”. As such, the sample size calculation for this study was based on Cohen’s convention for effect size - a difference between two means that equals to 0.5 x standard deviation is a “medium-sized” effect. With two paternal treatment groups and the assumption of normally distributed levels of folate and DNA
methylation, a treatment group size of 16 was required to have an 80% chance to detect a
difference equal in size to 0.5x standard deviation at $p < 0.05$ among the folate status and DNA
methylation means. Although there were no potential complications predicted for the dietary
intervention, an additional 4 mice per treatment group were included in the event of unexpected
animal death and for preliminary analyses of folate status. The litter clustering effect was taken
into consideration, so all outcome analyses took multiple pups by sex from one litter as a
repeated measure. For the results of gene-specific methylation, one male and one female from
each litter were taken. For all outcome analyses, the interaction between sex and diet was also
taken into account. We expected to successfully breed 36 pairs and find around 8 pups from
each litter.

Analysis of outcome measures

Statistical analyses were performed using SPSS 22.0 for Windows (SPSS, Chicago, IL). Continuous variables were log transformed due to deviation from normality. Body weights of male and female mice were compared over time using Student’s t test. Analyses of litter size and days to pregnancy were performed with the Mann Whitney U test and the Student’s t test, respectively. Plasma folate concentrations of sires and dams were analyzed using the Student’s t test, and the plasma and tissue folate concentrations of pups between the two paternal diet groups were compared using a mixed model with repeated measures analysis. Due to the fact that multiple pups of different sex were from the same litter, the clustering effect was accounted for with the use of the repeated measures analysis. These analyses of folate concentrations and gene-specific DNA methylation in pups included an interaction term between diet group and sex of pup to test whether the effect of the paternal diet depended on the sex of pup and vice versa. In the case of significant interactions, the analysis was followed by Tukey’s Honestly Significant difference post-hoc analyses to determine the nature of the interaction. All significance tests were two-sided and considered significant at $p < 0.05$. Results are expressed as mean ± SD or SE.
4.4 Results

4.4.1 Animal health and body weight

Health of the C57BL/6 mice was assessed daily, and they were in good health throughout the dietary intervention. Their body weights were recorded weekly and their growth curves were similar to the sex-specific standard curves provided by the vendor (Charles River Laboratories).

Growth curves were not significantly different between the dietary groups in males \( (p = 0.54, n = 20/\text{dietary group}) \) (Figure 4.4). Additionally, body weight was not significantly different \( (p > 0.05) \) between the dietary groups during each week of dietary intervention. There was no significant interaction effect of diet*time \( (p > 0.05) \) on body weight.

![Figure 4.4: Effects of folic acid intervention on C57BL/6 body weight. \( n = 20/\text{dietary group} \) (M: male), \( n = 40 \) (F: female).](image)

Figure 4.4: Effects of folic acid intervention on C57BL/6 body weight. \( n = 20/\text{dietary group} \) (M: male), \( n = 40 \) (F: female).
4.4.2 Litter size and days to pregnancy

There was no significant difference in the average number of pups per litter observed between the two paternal diet groups ($p = NS$; Table 1). However, the average number of days to pregnancy, which represents pregnancy efficacy, was significantly lower in the folic acid supplemented group ($p < 0.0001$; Table 4.4).

**Table 4.4: Effect of dietary folic acid on mating and pregnancy outcomes**

<table>
<thead>
<tr>
<th>Dietary Group (n)</th>
<th>2 mg FA/kg (13)</th>
<th>20 mg FA/kg (13)</th>
<th>P-value</th>
</tr>
</thead>
</table>
| Litter Size      | Mean +/- SD     | 5.2 ± 1.5        | 5.1 ± 0.8 | 0.9
|                  | Median [95% CI] | 5.0 [4.4, 6.0]   | 5.0 [4.6, 5.5] |
| Days to pregnancy| Mean +/- SD     | 5.3 ± 1.6        | 2.7 ± 1.1 | <0.0001
|                  | Median [95% CI] | 5.3 [4.6, 6.2]   | 2.7 [2.1, 3.2] |

1 P-value from Mann Whitney U test.
2 P-value from Student’s t test.

4.4.3 Plasma and tissue folate concentrations

Supplementation with 20 mg of folic acid per kg of diet resulted in significantly higher plasma folate concentrations in sires ($p < 0.0001$) compared with those that fed the control amount of folic acid (Table 4.5). As mentioned previously, female on the control diet of 2 mg of folic acid per kg of diet were randomly paired to the male mice at a ratio of 1:1 for breeding. Between the two groups of dams, there was no significant difference in plasma folate concentrations ($p < 0.05$) (Table 4.5).

Plasma and tissue folate concentrations of pups from sires that were on the folic acid supplemented diet were not significantly different from those of pups from sires that were on the control diet ($p > 0.05$) (Table 4.6). When taking the sex of the pups into consideration, there was no significant interaction between the sex and diet intervention for all measures of folate concentrations.
Table 4.5: Effect of dietary folic acid on plasma folate concentrations in sires and dams at necropsy

<table>
<thead>
<tr>
<th>Male Dietary Group (n)</th>
<th>2 mg FA/kg (13)</th>
<th>20 mg FA/kg (13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Folate of Sires (ng/mL)</td>
<td>Mean +/- SE Median [95% CI]</td>
<td>64.6 ± 7.6 61.8 [48.0, 81.3]</td>
<td>138.8 ± 11.6 142.6 [113.6, 164.0]</td>
</tr>
<tr>
<td>Plasma Folate of Dams (ng/mL)</td>
<td>Mean +/- SE Median [95% CI]</td>
<td>81.5 ± 12.3 65.5 [54.7, 108.3]</td>
<td>51.8 ± 6.5 55.6 [35.1, 68.4]</td>
</tr>
</tbody>
</table>

\(^1\)P value from Student’s t test.
The sires were on their respective diet for 3-4 weeks before breeding period.

Table 4.6: Effect of dietary folic acid on plasma and tissue folate concentrations in pups at necropsy

<table>
<thead>
<tr>
<th>Paternal Dietary Group (n)(^1)</th>
<th>2 mg FA/kg (13)</th>
<th>20 mg FA/kg (13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Folate (ng/mL)</td>
<td>Mean +/- SE Median [95% CI]</td>
<td>74.7 ± 19.1 53.2 [21.6, 127.8]</td>
<td>105.2 ± 17.1 119.5 [50.7, 159.6]</td>
</tr>
<tr>
<td>Hepatic Folate (µg/g of tissue)</td>
<td>Mean +/- SE Median [95% CI]</td>
<td>4.8 ± 0.5 4.4 [3.5, 6.0]</td>
<td>4.7 ± 0.7 4.8 [2.6, 6.9]</td>
</tr>
<tr>
<td>Colon Folate (ng/g of tissue)</td>
<td>Mean +/- SE Median [95% CI]</td>
<td>758.6 ± 148.1 905.0 [347.2, 1170.0]</td>
<td>899.1 ± 75.1 847.8 [660.0, 1138.1]</td>
</tr>
<tr>
<td>Kidney Folate (ng/g of tissue)</td>
<td>Mean +/- SE Median [95% CI]</td>
<td>663.5 ± 106.1 611.3 [368.8, 958.2]</td>
<td>672.1 ± 66.1 677.2 [461.7, 882.6]</td>
</tr>
</tbody>
</table>

\(^1\)n value refers to the number of litters from each group. Multiple pups from each litter were analyzed.
\(^2\)P value from Repeated Measures Mixed Linear Model Analysis.
4.4.4 Gene-specific DNA methylation

For the four genes we explored, the specific targeted CpG sites are shown in Figure 4.3. At birth, there were no significant differences in the CpG DNA methylation in the promoter region of the Ppar-α and Ppar-γ genes (p > 0.05; Figure 4.5A,B) between the two paternal dietary groups in the liver of the pups. There was no significant effect from the sex of the pups (p = NS), nor was there significant interaction between sex and dietary folic acid levels. The CpG DNA methylation in Igf-2DMR2 was significantly higher for female pups than male pups (p = 0.04); however, there was no significant effect from dietary folic acid levels (p = 0.8; Figure 4.6) or interaction between sex and dietary folic acid levels. Paternal folic acid supplementation significantly increased the CpG DNA methylation in exon 2 of the Gr gene (p = 0.04; Figure 4.7). There was no significant effect from the sex of the pups (p = NS), nor was there a significant interaction between sex and diet folic acid levels.

![Figure 4.5](image1.png)

**Figure 4.5:** DNA methylation at (A) Ppar-α (3 CpG sites) and (B) Ppar-γ (5 CpG sites) of liver in pups at necropsy. n=16 (2 mg FA/kg); n=18 (20 mg FA/kg), p_A = 0.1, p_B = 0.1 from a Repeated Measure ANOVA.
Figure 4.6: DNA methylation at *Igf-2* (5 CpG sites) of liver in (A) male and (B) female pups at necropsy. n=16 (2 mg FA/kg); n=18 (20 mg FA/kg). Effect of diet, \( p = 0.8 \); sex, \( p = 0.04 \) from a Repeated Measure ANOVA.

Figure 4.7: DNA methylation at *Gr* (8 CpG sites) of liver in pups at necropsy. n= 16; \( p = 0.04 \) from a Repeated Measure ANOVA.
4.5 Discussion

Recently reported associations between paternal folate status – both deficiency and supplementation - and DNA methylation in sperm and offspring tissues point to a novel complex relationship that may have important implications for fetal development and growth and offspring’s health (451–453,455). We investigated the effects of paternal folic acid supplementation on plasma and tissue folate concentrations and hepatic gene-specific DNA methylation levels of the offspring using a C57BL/6 mouse model. We additionally assessed the effects of paternal folic acid supplementation on pregnancy outcomes.

Amino-acid defined diets of 2 (control) or 20 (supplementation) mg folic acid/kg altered the folate status of the sires, thus the dietary interventions were effective in modulating the plasma folate concentrations in the two groups. We observed significantly higher plasma folate concentrations in the supplemented group relative to the control. When we compared the plasma folate concentrations of the two groups of female mating partners of the males, we detected no significant difference. This observation was as expected since all dams were maintained on the control diets. The plasma folate concentrations of the dams were similar to those of the sires on the control diet, and the range of plasma folate concentrations observed in our study was consistent with previous studies using similar folic acid supplementation in mice (283,483). We used a 20 mg folic acid/kg diet, which is tenfold of the basal amount of folic acid required for rodents. Such intake levels of folic acid are not commonly seen in the North American population, although individuals with specific medical conditions may be recommended such levels of folic acid. Compared to humans, the biotransformation of folic acid by DHFR in rodents is more rapid, and higher levels of folic acid intake in rodents are validated to induce similar physiological effects in humans.

An increasing amount of evidence points to the possible influences of paternal nutritional and environmental factors on the characteristics and epigenome of sperm, with subsequent consequences in mating, pregnancy and offspring health (411–413,420,484). With respect to paternal folate intake, Lambrot et al. reported significant reduction in pregnancy rate and increase in post-implantation loss in the folate deficient group (0.3 mg folic acid/kg) compared to the folate sufficient group (2.0 mg folic acid/kg) (455). Although they did not detect a difference in mating behavior, they did observe an increase in breeding interval in females bred to the folate deficient males. To our knowledge, our study is one of the first to
examine mating patterns and pregnancy outcomes in a rodent model with folic acid supplementation. We assessed the litter size and breeding interval until pregnancy was first detected by plug-positive females. Although there was no significant difference in the litter size between the control and supplementation groups, there was a significant difference in breeding interval until detection of pregnancy. Consistent with the findings of Lambrot et al., the breeding interval for the control group was longer than the supplementation group (455). The greater breeding interval in the control group in comparison to the supplementation group may be explained by the existing evidence for the negative effects of folate deficiency on male fertility. Human studies have shown an inverse relationship between total daily folate intake and the frequency of aneuploidy sperm (404), and also demonstrated a correlation between folate deficiency and decreased sperm count and increased sperm DNA damage (430,450). More recently, studies using mouse models have reported that the use of methotrexate and antifolate chemotherapeutics is associated with decreased sperm count and increased sperm DNA damage (485,486), as does folate deficiency (0 mg folic acid/kg) (449).

In pups, we did not find significant differences in plasma, hepatic, colon and kidney folate concentrations between the control and supplementation groups. This is inconsistent with the results of Kim et al. that reported significantly lower folate levels in fetal liver and placenta in pups sired by folate deficient rats (0 mg folic acid/kg) than those sired by folate supplemented (8 mg folic acid/kg) rats (452,453). The duration of the dietary interventions in these two studies was 4 weeks, which is similar to that of our study (3-4 weeks). Moreover, the point of tissue collection was similar, where our endpoint was the time of birth, and the fetuses in their studies were killed at day 20 of gestation (452,453), which marks the end of the typical gestational period of both rats and mice. One possible explanation for our results is that the potential effects of paternal folic acid intake on the pups’ plasma and tissue folate concentrations may be “corrected” by fetal exposure to maternal folic acid intake throughout the gestational period. A future study with 3 different endpoints for the pups’ tissue collection (day 7, 14, 21 of gestation) can be used to observe whether there are marked effects of paternal folate intake on the pups at the start of gestation and, if there are significant effects, how the intrauterine environment modulates those effects during different time windows of gestation. As previously discussed, the time between fertilization and implantation is marked by genome-wide demethylation, and the period of time onwards from implantation is marked by de novo methylation and maintenance methylation and demethylation. However, the critical window for DNA methylation changes
specific to folic acid supplementation has yet to be clearly established. In an accepted
manuscript, Ly et al. conducted a study with this design for the effects of maternal folic acid
supplementation at various gestational periods on folate levels, DNA methylation and gene
expression in the rat offspring (487). They reported significant time- and tissue-specific effects
on the pups, where maternal folic acid supplementation during the 2\textsuperscript{nd} or 3\textsuperscript{rd} week of gestation or
throughout pregnancy significantly increased their brain folate concentrations, however only
maternal supplementation all throughout pregnancy significantly increased liver folate
concentrations. Another theory that may additionally explain our results is that perhaps the more
permanent and resistant methylation patterns in the sperm epigenome is established earlier on in
the embryonic development of the sire, during the process of differentiation of primordial germ
cells into spermatogonia. Perhaps the DNA methylation patterns acquired after birth of the sire
from environmental exposures are more susceptible to the genome-wide demethylation that
occurs after implantation of the fertilized ovum. In order to explore this hypothesis, a
multigenerational study similar to that by Lambrot et al. would be useful. The complex
relationship between maternal folic acid supplementation and the folate status of the offspring is
not yet clearly elucidated, but nonetheless points to a potential gestation- and tissue-specific
effects of paternal folic acid supplementation that have yet to be explored. It would be

Of the genes we explored for hepatic gene-specific methylation in pups, $Gr$ methylation
was significantly higher in the supplementation group compared to the control group. There
were no significant differences in methylation levels for $Ppar$-$\alpha$, $Ppar$-$\gamma$ and $Igf$-2 between the
groups, although there was a significant effect of the sex of pups on $Igf$-2 methylation. An
inverse relationship between DNA methylation in the regulatory regions and gene transcription
is not always the case, and decreased DNA methylation in the promoter region of $Gr$ has
previously been shown to decrease $Gr$ expression the same mouse model (475). While the
functional significance of the difference in methylation levels observed in this study remains
widely unknown, there are potential implications of altered $Gr$ methylation and expression. The
action of the glucocorticoid hormone is dependent on $Gr$ transcription, where the amount of
glucocorticoid binding to the receptor is determined by levels of the mRNA expression (488).
Thus, the difference in methylation levels of $Gr$ observed in this study potentially impacts the
downstream effects involved in not only fetal growth and development, but also hypertension,
gluconeogenesis and physiologic response to insulin (489). In fact, previous maternal studies
have shown that maternal dietary factors influence $Gr$ expression, as they reported increased $Gr$
expression in fetal tissues from protein restricted maternal diet and treatment of pregnant rats with dexamethasone (490,491).

As discussed earlier, Igf-2 was chosen in the present study because it is a fetal growth factor and an imprinted gene that is more greatly expressed from the paternal allele (478). Although we did not analyze gene expression, a recent study using a rat model to examine the effects of paternal folate deficiency on the hepatic expression of Igf-2 in the offspring, where there were no significant differences found between the folate deficient and sufficient groups (454). In the present study, the level of Igf-2-methylation in the female pups was significantly greater than that in the male pups \((p = 0.04)\). Altered methylation of this gene has been linked to imprinted disorders, such as the Beckwith-Wiedeann syndrome (492), and selective CpG methylation of this gene during infancy has been associated with reduced hepatic expression and the development of fatty liver and impaired glucose metabolism later in life (493). Because the difference in DNA methylation levels between the sexes is less than 1%, this raises the question about its biological significance. In fact, human studies have shown that the level of DNA methylation of imprinted genes is generally equal between the sexes (494). However, a seemingly insignificant difference in methylation can greatly influence gene expression. Begum et al. demonstrated that a decrease in hypothalamic NRC31 DNA methylation even of 0.3% was associated with a five-fold increase in mRNA expression (495).

Existing data on the effect of paternal folate intake on gene-specific methylation in the offspring are very limited and inconsistent. A few studies have assessed global methylation and gene expression, but only Lambrot et al. have additionally examined promoter methylation of imprinted genes (455). They reported 57 differentially methylated genomic regions between sperm from the folate deficient and sufficient groups (455). In the folate deficient group, methylation levels were higher for some genes and lower for others. The affected promoter regions were of genes implicated in development, functions in the central nervous system, kidney, spleen, digestive tract and muscular tissue. They were also associated with the development of chronic diseases, including diabetes, autoimmune diseases, neurological conditions, autism, schizophrenia and cancer (455). However, DNA methylation of the 65 imprinted genes assessed in this study had no significant changes in promoter DNA methylation (455). When the functional consequences of these methylation results were examined, only two genes (Cav1 and Txndc16) from the folate deficient males had differential expression in the placenta along with being differentially methylated in sperm (455). These genes were Cav1, a
cell cycle regulator, and \textit{Txndc16}, a gene involved in cell homeostasis. This study demonstrates the susceptibility of the intrauterine environment to the epigenetic reprogramming of sperm DNA, however additionally shows that the changes may not amount to any significant functional implications for the developing offspring. Hence, the increase in DNA methylation we observed for \textit{Gr} in the liver of the pups with FS sires, although statistically significant, may not translate to differential expression in the offspring.

To address the dietary interventions of the present study more specifically, we chose the mode of supplementation to be in the diet rather than in the form of an injection. Although folic acid injections are administered to patients with anemia caused by low folate diet, pregnancy, alcohol use, liver disease or dialysis (496), the interest of the present study was in the effect of the mandatory folic acid supplementation in dietary sources in North America on the offspring. The dietary intervention is more relevant to the aims of this study than a folic acid injection because it serves to parallel the daily dietary intake and metabolism of folate in humans.

In a standard chow diet, although it is composed of natural ingredients, such as cereal and milk powders, the disadvantage of adding vitamins and minerals to this type of diet is that the components can easily vary, depending on the state of the cereal parts from factors such as harvest conditions and contaminants (497). Although amino acid-defined diets are costly, all the ingredients are chemically well-defined and under rigid quality control. Hence, we utilized this type of diet so that only the folic acid content would be altered in the two paternal dietary interventions. However, the amount of amino acids in the administered diet that is far greater than the level of folic acid contained raises the question of whether the differences observed in this study are biologically significant. For example, there was 8.2 g methionine/kg diet, compared to the 2 and 20 mg folic acid/kg diet for the control and supplemented diets. Although we are assuming that the observed differences are due to the folic acid content in the diet, it is important to keep in mind that methionine directly generates SAM for DNA methylation (23). It would be wise to decrease the amount of other 1-carbon nutrients in the administered diet, so that the effects of folic acid in the paternal diet on the offspring’s folate levels and DNA methylation can be more distinguished.

We initially considered having two levels of folic acid in the maternal diet (2 and 5 mg folic acid/kg of diet) and four levels of folic acid in the paternal diet (0, 2, 10 and 20 mg folic acid/kg of diet) in order to examine interaction effects from modulating the amount of folic acid
in the maternal and paternal diets. However, following sample size calculation with 80% power to detect a medium effect, the sample size we needed was 576 pups. This number was not feasible, considering the available resources, time and one’s capacity to carefully handle all of the mice. Therefore, we decided to approach this study as a proof-of-concept study by keeping the maternal diet on a single level of folic acid, 2 mg folic acid/kg. Although choosing 20 mg folic acid/kg would have been more relevant and reflective of current folate intake of pregnant women in the population, Mejos et al. have recently reported effects of concurrent maternal and paternal folic acid supplementation on increasing hepatic global DNA methylation (454).

Because we were interested in the independent effect of paternal folic acid supplementation on the offspring and there was a possibility that the paternal effects may be obscured by those from the maternal diet, we chose the maternal control diet to be 2 mg folic acid/kg diet. For the levels of folic acid in the paternal diet, since there were a number of studies examining the effects of paternal folate deficiency (452–454) and only a few assessing the effects of paternal folate supplementation (451), we decided on 2 (control) and 20 (supplemented) mg folic acid/kg of diets to explore folic acid supplementation and compare it to a baseline level. We believed that future studies could take an additional diet group of 0 mg folic acid/kg and assess the effects of paternal folate deficiency and supplementation by comparing it to the 2 mg folic acid/kg group.

We measured plasma folate concentrations in the sires, dams and offspring because our laboratory had not developed a standardized microbiological assay for RBC folate concentrations. Nonetheless, we fasted the mice for 2 hours prior to sacrifice in order to minimize variation in plasma folate concentrations from their dietary intake before sacrifice and sample collection. For future studies, it is very important to measure RBC folate concentrations because plasma folate concentrations are more indicative of short-term dietary intake and supplement use, while RBC folate concentrations are more representative of long-term folate tissue stores (27). RBCs uptake folate only during their maturation in bone marrow, and their turnover rate of 120 days minimize the short-term variations of folate levels from dietary and supplemental intake.

Nutritional epigenetic studies widely assess both global and gene-specific DNA methylation. We chose to focus on the latter because, although global DNA methylation is a good indicator for the effects of nutritional factors on genome-wide DNA methylation levels, the measure does not identify the affected genes are affected or point to potential implications of the modulated DNA methylation levels on the health of the offspring. For gene-specific DNA
methylation, we selected these genes for their readily available gene sequences and previously reported changes in DNA methylation due to maternal folic acid supplementation. However, the mechanism by which maternal folic acid supplementation alters DNA methylation patterns in the offspring may not be the same for paternal folic acid supplementation. Hence, the genes affected by paternal folic acid intake may be unrelated to those identified through maternal folic acid studies. Since the mechanisms behind the epigenetic paternal effects on the offspring epigenome have yet to be thoroughly explored and described, we note that a more appropriate method of observing paternally-dependent effects on DNA methylation in the offspring would be a methylation array, which is not yet available for mice, instead of a targeted DNA methylation analysis. Similar to the reports by Lambrot et al., the data would help to identify more general classes of genes that are affected by the amount of paternal folic acid intake (455). With the information from these studies, we can move to an approach of targeting specific genes and quantifying the modulation in DNA methylation levels and its functional consequences in gene expression.

We were primarily interested in assessing gene-specific DNA methylation in the liver because this tissue contains the highest portion of total body folate and is the main site for storage of folate taken up by hepatocytes (437). Initially, we considered additionally analyzing gene-specific DNA methylation in the placenta, brain and sperm. Collecting the placenta for the current study was not possible because we could not predict the exact time of birth of the pups to obtain the placenta, and the only other means to collect the tissue would have been prior to birth. For the brain, we conducted a pilot study prior to the present one in order to practice the skills needed to monitor the breeding period and confirm pregnancy. Another aim of the pilot study was to see whether or not the collection of the brain of the pups was feasible, however the pups’ skull and tissue were too fragile for collection. As Lambrot et al. included in their study, it is very important for subsequent studies to obtain the sires’ epididymides and homogenize them in solution to count the sperm heads using a hemocytometer (455). This sperm count and its other characteristics will be useful in providing explanations for the observed changes in breeding outcomes.

Our examination of the effects of paternal folic acid supplementation on hepatic gene-specific methylation levels in the offspring is limited by high sample-to-sample variability from a small sample size. Since we were also limited by the tissue size to observe the functional significance of the gene-specific methylation results, future analyses should be conducted to
measure protein expression levels and downstream effects. Furthermore, future studies assessing the effects of paternal folic acid supplementation on folate levels and gene-specific methylation levels in sperm may contribute to explaining the effects of supplementation on male fertility. These observations using the sperm epigenome may help to explain the possible retention of certain methylation patterns in the sperm and their transfer down to the offspring.

Observations from the gene-specific methylation are not only limited by the lack of protein expression analysis for determining their functional significance, but also insufficient to make conclusive remarks because the number of CpG sites we explored were very few. While we chose 3 CpG sites for *Ppar-α*, 5 for *Ppar-γ* and *Igf-2*, and 8 for *Gr*, there are actually 120, 97, 234 and 190 known CpG sites for the respective genes. Clearly, the observations made on a small fraction of the total number of CpG sites would not accurately represent the effects of paternal folic acid supplementation on the methylation levels at these regions. However, we chose CpG sites that are either at or near promoter regions – sites that would affect gene expression. With the significant increase in DNA methylation we observed for *Gr* with paternal folic acid supplementation, it may be worthwhile to assess the functional consequences of the increase in DNA methylation with protein expression analysis in a future study.

An additional potential gene candidate for gene-specific methylation in similar future studies is *Mtfr*. This gene encodes for MTHFR, which is responsible for converting 5,10-methyleneTHF to 5-methyleneTHF, the primary circulating form of folate that provides a methyl group for methionine synthesis. Five to 20% of males are expected to exhibit the *MTHFR C677T* polymorphism, which affects their folate status. *MTHFR* expression is highest in testes, where DNA methylation activity is markedly changing with sperm development and epigenetic programming. The polymorphism, *MTHFR C677T*, has been linked to an increased risk of disease, poor outcomes for chronic disease, reproduction and cancer (455).

### 4.6 Conclusion

In the current study, there was a significantly shorter period of breeding until detection of pregnancy for dams whose male breeding partner was on the supplemented diet. This effect suggests the possible advantageous role of folic acid supplementation on breeding patterns, and is consistent with previous findings of a significant association between folate deficiency and negative pregnancy outcomes by Kimmons et al (20). Although paternal folic acid supplementation of a tenfold increase from the control was reflected in the significantly higher
plasma folate concentrations of the sires, this effect was not observed in the plasma or tissue folate concentrations of the pups. This may suggest that the supply of the maternal diet (2 mg FA/kg) during the three weeks of gestation for the developing fetus is more predictive of the folate content in the plasma and tissues of the offspring at birth. The significantly greater CpG DNA methylation for Igf-2 in the liver of female pups compared to the male pups suggests an inherent difference in methylation levels due to the sex of the pups. Although there was a significantly greater level of CpG DNA methylation for Gr in the liver the pups from the paternal supplemented group, the functional ramifications of this effect is unclear unless the expression of this gene is measured.
Chapter 5: General discussion, future directions and conclusion

5.1 Summary and general discussion

A dietary intervention study with a C57BL/6 mouse model was utilized to investigate the effects of paternal folic acid supplementation on the folate levels and DNA methylation levels in the pups. We observed the effects of folic acid supplementation on the DNA methylation levels of specific genes, including Ppar-α, Ppar-γ, Gr and Igf-2, of the pups. We measured the folate levels of the sires, and observed that the dietary folic acid intervention (2 mg folic acid/kg and 20 mg folic acid/kg) was effective in modulating their plasma folate concentrations prior to mating.

Significantly lower number of mating days to pregnancy was observed in mating partners with the male mouse supplemented with folic acid, relative to control. The higher number of mating days to pregnancy in the control group (supplemented with 2 mg folic acid/kg) is consistent with previous studies that have noted the negative effects of folate deficiency on pregnancy outcomes and sperm characteristics (449,450). There were no significant differences observed between the control and supplementation groups for the litter size. Therefore, the data suggest the possibility that higher paternal folate levels improve sperm characteristics, such as sperm count and motility, consequently resulting in a shorter mating period. Albeit, the results from this study and the existing evidence for the potential effects of folic acid supplementation on improving fertility are inadequate to make health policy recommendations for the public. However, it is important for individuals (men and women) considering pregnancy to be aware of and acknowledge the rising interest in and the current evidence that points to the potential contribution of paternal nutritional and behavioral factors on sperm, in addition to the consequences of the offspring are. In fact, current recommendations for folic acid supplementation to women in the reproductive age group (12-45 years of age) in Canada take not only the risk of the women for NTDs or other congenital abnormalities into consideration, but also the risk of the male partner for these conditions (498). For example, women with a low risk for NTDs or other anomalies related to folic acid and a low risk male partner are recommended to consume folate-rich foods and a daily oral multivitamin supplement with 0.4 mg folic acid for a minimum of 2-3 months prior to conception, throughout pregnancy and for 4-6 weeks postpartum. However, women with a high risk for NTDs or other folic acid-related congenital anomalies with a male partner with a history of NTD are recommended to consume
folate-rich foods, a daily multivitamin supplement with 0.4 mg folic acid for a minimum of 3 months before conception until the 12th week of gestation. Thereafter until 4-6 weeks postpartum or onto breastfeeding, she should take a daily multivitamin containing 0.4 mg to 1.0 mg folic acid (498). Such studies as the present one can potentially lead to forming recommendations of periconceptional folic acid supplementation for women in the reproductive age group which take the male partner’s folate intake and levels into consideration.

No significant differences were observed for the pups’ plasma, liver, kidney and colon folate concentrations between the control and supplemented groups. However, in a similar study conducted by Kim and colleagues (453,454), they observed marked differences in the liver folate content of pups sired by folate-supplemented and folate-deficient mice. The data suggests the possibility that, although the paternal plasma folate concentrations were significantly different between the two groups, either the difference was not enough to result in marked differences in the pups’ folate concentrations, or the 3 weeks of gestation in the maternal uterine environment (dams receiving 2 mg folic acid/kg) reversed the paternal effects on the pups’ folate concentrations.

Although we did not observe significant effects of paternal folic acid supplementation on the CpG DNA methylation of Ppar-α, Ppar-γ, and Igf-2 in the pups, there was a significant effect of sex on the methylation of Igf-2 and of folic acid supplementation on the methylation of Gr. Since the CpG sites chosen for our analysis of gene-specific methylation were very few compared to the total number of available CpG sites for these genes, there is a need for further studies exploring the additional sites.

There has been a recent increase in the number of studies that investigate the consequences of paternal nutrition and habits related to health on their offspring. The paternal characteristics which these studies have focused on include the intake of different foods, such as fat and various vitamins, BMI, smoking and their level of physical activity (400,402,405). They observed their effects on the risk of the offspring to develop chronic diseases, such as diabetes (22,402) and cancer (405), have a higher risk for obesity (22,418) and exhibit changes in their gene methylation and expression (411–413). Only a handful have specifically investigated the effects of paternal folic acid intake and folate levels on the offspring’s health and epigenetic outcomes (451,454,455). Therefore, there is a need for additional studies that consider the paternal contribution of the effects of folate intake and levels to the development and health
consequences of the offspring. In fact, the significantly lower number of mating days to pregnancy in the folic acid supplemented group and the marked differences in the methylation of few of the selected genes suggest that there is a significant role of paternal folic acid supplementation on mating outcomes and the epigenetic programming in the offspring. With the evidence from other studies, this relationship can be further explored and articulated in order to incorporate dietary folic acid recommendations for males, in addition to the existing guidelines for females.

5.2 Future directions

As explained before, due to the variability between experimenters in many folate studies, the a priori sample size calculation was based on an existing convention that a difference between two means which is equivalent to 0.5 of the standard deviation is indicative of a significant “medium” effect size. For the future, not only would a greater sample size allow for the detection of a significant “small” effect size, but it would also be useful to determine, from existing literature, the range of paternal folate levels beyond which significant effects on fertility and DNA methylation of important genes are observed.

Although the homology between humans and mice, and that between humans and rats are similar, we chose a mouse model because we considered the possibility of conducting a microarray to assess functional outcomes of changes to DNA methylation in the offspring, and microarrays for mice are not yet available. Considering a rat model for a future study similar to the present one is important because rats have tissues of greater size, hence smaller and more fragile tissues, such as the epididymides, prostate, brain and spleen will be easier to collect. In fact, the rat model may potentially be a better choice for future studies that aim to observe the effects of paternal folic acid supplementation on the offspring’s health outcomes, such as cardiovascular disease, cognitive function and diabetes, because the physiology of rats is easier to monitor than that of mice (499).

Future studies should investigate the relationship between paternal folic acid supplementation and sperm characteristics, including sperm motility, count, DNA fragmentation and sperm epigenome. Although the number of such nutritional epigenetics studies are inadequate for a consensus, Chavarro et al. and Jensen et al. have observed a nonsignificant correlation that suggests obese men with a BMI greater than 25 kg/m² have lower total sperm count than men with normal BMI (391,392). Moreover, Hofny et al. and Hammoud et al. have
observed a negative correlation between BMI and sperm motility (388,389). DNA fragmentation is normal in men at a level of 3-5% of sperm, however, it is indicative of a greater risk of male infertility if the level is 25-30% of sperm (386). DNA fragmentation index (DFI) indicates the proportion of sperm in a sample of semen with single or double strand breaks. Men with a BMI greater than 25 kg/m² (386) or with type 2 diabetes (500) have shown to have greater sperm DFI and other structural defects in sperm. Investigating these characteristics of sperm, the only paternal contribution to the developing zygote, will hopefully provide information to explain the significant difference we observed in the breeding period prior to a successful pregnancy. Additionally, similar to the study by Lambrot et al. (455), it would be notable to explore other outcome measures of fertility, such as embryo loss and development at various points in the gestational period, and embryo weight and length. Such approach can help to examine the relationship among paternal folate supplementation, its effects on the sperm, and the consequent implications for the growth and development of the offspring. Finally, since there also is evidence pointing to the role parental folate status plays in the development of the neural tube and craniofacial structures (455), future studies can consider histopathological analysis to examine other phenotypic characteristics of the offspring, including facial abnormalities, limb defects, bone formation, and assess the progression of their skeletal development throughout the gestational period and beyond. Since the present study focused on the effects of paternal folic acid supplementation on the offspring’s DNA methylation of key genes in their growth and development at birth, the blood and tissue collection that had to occur immediately after birth made conditions difficult to consider and measure these other outcomes. In the future, it would be useful to consider collaborating with a group with the resources and skills to conduct a histopathological analysis on the fetuses in order to explore the phenotypic and growth outcomes mentioned above.

Studies such as the one previously mentioned of Lambrot et al. have utilized rat models to perform a microarray to identify genes that had altered levels of expression from paternal dietary interventions (452,455). For future gene-specific DNA methylation studies, these existing data will be helpful in selecting important genes, including other imprinted genes, and their specific CpG sites to support or refute the rising concern of the modulating effects of paternal diet on DNA methylation and gene expression in the offspring. In fact, researchers may consider performing an array first to identify genes with altered levels of expression, and
thereafter proceed with CpG-methylation analysis to locate sites with altered levels of methylation.

Although we did not observe significant differences in plasma and tissue folate concentrations of pups in the control and supplemented groups, we suggested that this effect may have been due to possible reversal effects in the intrauterine environment during the three weeks of gestation, with the maternal diet maintained at 2 mg folic acid/kg. Plasma folate concentrations should be assessed as early as the size of the fetus is significant enough to be obtained from the placenta in order to observe the effects of paternal folic acid intake and minimize the possible confounding effects of maternal folic acid intake during gestation. If there is a difference between the control and supplemented groups, it may then be useful to compare the plasma concentrations of some of the fetuses at their second week of gestation with those at birth to identify the gestational period most susceptible to the effects of both maternal and paternal folic acid diets. In a recent study by Ly et al., female rats were fed a control diet throughout pregnancy, folic acid supplementation at the 1st, 2nd or 3rd week of gestation, or folic acid supplementation throughout pregnancy (487). Their objective was to assess the time-dependent effects of folic acid supplementation on the pups’ tissue folate concentrations, DNA methylation and gene expression. They reported a significant increase in the pups’ brain folate concentrations with folic acid supplementation during the 2nd and 3rd weeks of gestation, and a significant increase in the pups’ liver folate concentrations with supplementation throughout pregnancy. They also observed a significant decrease in the pups’ brain global DNA methylation as supplementation occurred during early to late gestation, and a significant decrease in the pups’ hepatic expression of Er-α, Gr and Ppar-α with supplementation in late gestation or throughout pregnancy (487). Their reports suggest that the effects of maternal folic acid supplementation during pregnancy on the pups’ tissue folate concentrations, DNA methylation and gene expression are more prominent for the later stages of gestation and are tissue-specific. Therefore, from the evidence that supports the potential time- and tissue-specific effects of maternal folic acid supplementation, it is worthwhile to assess these effects for paternal folic acid supplementation as well.

With rising evidence of paternal epigenetic contributions to the offspring, the interaction between maternal and paternal folic acid supplementation should be assessed. Mejos et al. have used a rat model study to investigate the effects of maternal and paternal folate deficiency and supplementation on the hepatic folate concentrations, global DNA methylation and gene
expression in the pups (454). After breeding, they had four groups of litters – supplemented sires with supplemented dams (PSxMS), supplemented sires with deficient dams (PSxMD), deficient sires with supplemented dams (PDxMS) and deficient sires with deficient dams (PDxMD). They reported significantly lower hepatic folate content in the pups of the PDxMD, PSxMD and PDxMS groups compared to the PSxMS group and no significant differences in the hepatic expression of Fr-α, Igf-2 and Igf-1r among the four groups (454). Their results suggest that the hepatic folate content of the offspring may significantly decrease if either of the parental folate intake is low. Assessing the parental effects of folic acid supplementation will be useful in clearly elucidating the extent of the contribution of maternal and paternal folic acid supplementation on the plasma and tissue folate content and epigenetic outcomes of the offspring.

An interesting consideration for future studies is the potential switch from administering folic acid to 5-methylTHF. To our knowledge, there are no animal or human studies comparing the effects of 5-methylTHF and folic acid on the epigenetic outcomes of the offspring. Because there was no available stable form of 5-methylTHF to incorporate into rodent diets, folic acid has been and is currently being utilized in rodent models. With a stable form of 5-methylTHF now available, researchers are beginning to compare folic acid and 5-methylTHF. Although folic acid supplementation studies are relevant for the intake of folic acid-containing supplements and supplemented wheat and grain products in the population, studying the effects of 5-methylTHF will shed light on the effects of parental natural folates on the folate levels and DNA methylation in the offspring. If it is observed that there are significantly different effects of folate intake in the form of natural folates or folic acid on the offspring’s folate levels, DNA methylation, and gene expression, then it will also be important to study the implications on the health of the offspring and consider the possibility of replacing foods and vitamins currently supplemented with folic acid with 5-methylTHF supplementation. If there are such differences in supplementation with folic acid versus 5-methylTHF, then an additional concern would be to determine new recommendations for women of child-bearing age regarding their folate or folic acid intake.
5.3 Conclusion

Our data contribute to the evidence recently reported for the support of the effects of paternal folic acid intake on breeding outcomes and gene-specific methylation. The functional implications of the increase in methylation of Gr for the pups in the supplemented group should be further explored with protein expression analyses. Additionally, our data suggest that paternal folic acid supplementation may improve factors of fertility, and the effects of paternal supplementation on characteristics of sperm should be assessed in future studies. Because our data are among the first to address paternal epigenetic effects that were long disregarded, they will serve as a foundation, along with the evidence from similar recent studies, for many more investigations of parental nutrigenomics. The data and future work will help to address the current dietary folic acid recommendations and fortification mandate, possibly modifying them for both females and males in the population once this area of interest is further investigated.
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Stress, Epigenetics, and Alcoholism

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