COMPARATIVE IN VITRO STUDIES OF FOLIC ACID VERSUS 5-METHYLTYRHYDROFOLATE SUPPLEMENTATION IN HUMAN COLORECTAL CANCER CELLS

by

Hea Jin Cheon

A thesis submitted in conformity with the requirements for the degree of Masters of Sciences Graduate Department of Nutritional Sciences University of Toronto

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COMPARATIVE IN VITRO STUDIES OF FOLIC ACID VS. 5-METHYLTERETRAHYDROFOLATE SUPPLEMENTATION IN HUMAN COLORECTAL CANCER CELLS

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Abstract

High folate status from high folic acid (FA) intakes may have unintended and serious deleterious effects on human health, such as increased cancer progression and epigenetic changes. Some studies suggest that supplementation with 5-methyltetrahydrofolate (5MTHF) may be a safer means of providing folate supplementation. We compared the effects of FA versus 5MTHF supplementation on cellular growth, intracellular folate concentrations, and expression of genes involved in folate metabolism and one-carbon transfer reactions in human HCT116 and Caco-2 cell lines. 5MTHF supplementation generally resulted in significantly faster cellular proliferation and higher intracellular folate concentrations in both cell lines. 5MTHF supplementation also resulted in higher global DNA methylation in Caco-2 compared to FA, while the opposite occurred in HCT116. No conclusive comparative effects of FA versus 5MTHF supplementation on gene expression were observed. Future studies are warranted to determine which form of folate is a more appropriate and effective for improving folate status.
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<table>
<thead>
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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>5MTHF</td>
<td>5-methyltetrahydrofolate</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine preceding guanine</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>CT</td>
<td>cycle to threshold</td>
</tr>
<tr>
<td>DHF</td>
<td>dihydrofolate</td>
</tr>
<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegration per minute</td>
</tr>
<tr>
<td>dTMP</td>
<td>deoxythymidine monophosphate or thymidylicate</td>
</tr>
<tr>
<td>dUMP</td>
<td>deoxyuridine monophosphate</td>
</tr>
<tr>
<td>FA</td>
<td>folic acid</td>
</tr>
<tr>
<td>FPGS</td>
<td>folylpolyglutamate synthase</td>
</tr>
<tr>
<td>FR</td>
<td>folate receptor</td>
</tr>
<tr>
<td>GCPII</td>
<td>glutamate carboxypeptidase II</td>
</tr>
<tr>
<td>GGH</td>
<td>γ-glutamyl hydrolase</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine monophosphate</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine monophosphate or inosinic acid</td>
</tr>
<tr>
<td>MAT</td>
<td>methionine S-adenosyltransferase or SAM synthase</td>
</tr>
<tr>
<td>MBD2</td>
<td>methyl binding protein 2</td>
</tr>
<tr>
<td>MS</td>
<td>methionine synthase</td>
</tr>
<tr>
<td>MTHFR</td>
<td>methylene tetrahydrofolate reductase</td>
</tr>
<tr>
<td>MTHFRD</td>
<td>methylenetetrahydrofolate dehydrogenase</td>
</tr>
<tr>
<td>MTRR</td>
<td>methionine synthase reductase</td>
</tr>
<tr>
<td>NTD</td>
<td>neural tube defect</td>
</tr>
<tr>
<td>PABA</td>
<td>para-aminobenzoic acid</td>
</tr>
<tr>
<td>PCFT</td>
<td>proton coupled folate transporter</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RFC</td>
<td>Reduced folate carrier</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SHMT</td>
<td>serine hydroxymethyltransferase</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>tHcy</td>
<td>total homocysteine</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofolate</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>UL</td>
<td>upper tolerable intake</td>
</tr>
<tr>
<td>UMFA</td>
<td>unmetabolized folic acid</td>
</tr>
</tbody>
</table>
Chapter 1  Introduction

Folate is a B vitamin involved in one-carbon transfer reactions including nucleotide biosynthesis and biological methylation reactions (1). Folate plays an important role in human health and disease as its deficiency is linked to megaloblastic anemia, birth defects, cognitive impairment, cardiovascular disease, and cancer (1). Folic acid (FA; the synthetic form of folate) supplementation has been promoted as an effective nutritional strategy to treat and/or prevent these disorders. As a public health initiative, mandated FA fortification in North America succeeded in improving folate status and reducing rates of neural tube defects (NTDs) (2). The current RDA for adults is 400 μg/day dietary folate equivalent (DFE) (1). It is estimated that the Canadian population consumes 200 μg/day from natural dietary sources and 100-200 FA μg/day from fortified sources in the post-fortification era (2). When supplementation is taken into consideration, an individual can consume even higher amounts of folate in the form of FA. In the United States (US), approximately 50% of adults or children who regularly consume supplements exceed the tolerable upper limit (UL) for FA (1mg/day) (3). A study by Colapinto et al. found that, in Canadians, the prevalence of folate deficiency is extremely low (<1%), and that >40% of Canadians had red blood cell (RBC) folate concentrations above the level considered “high” (>1360 nmol/L) (4).

Emerging evidence, primarily from animal models, suggests that high intakes and blood levels of folate/FA may have serious deleterious effects on human health including tumor promotion and epigenetic instability (5). FA can saturate the key enzyme (dihydrofolate reductase, DHFR) involved in FA biotransformation (6), leading to the appearance of unmetabolized folate (UMFA) in the circulation. FA supplementation and high levels of UMFA have been purported to disturb the folate metabolic and one-carbon transfer pathways (5, 7), but
it is unclear whether these adverse metabolic effects are unique to high levels of FA or high folate status in general (3). It is speculated that FA, but not natural folate, may be responsible for these adverse health effects and that supplementation with 5-methyltetrahydrofolate (5MTHF), the natural and primary circulating form of folate, may be a safer means to provide supplemental levels of folate (3).

Clinical studies (refer to Table 2.9) have shown that 5MTHF supplementation is as effective as FA in improving blood folate levels and reducing plasma concentrations of homocysteine, a reliable inverse indicator of folate status (8). However, studies comparing the effects of FA and 5MTHF supplementation on folate metabolism and intracellular one-carbon transfer reactions are lacking at present. In addition, while the UL for FA is widely established, there is no established UL for 5MTHF. Before 5MTHF supplements were commercially available, a UL for natural folates was not necessary because it was very difficult to achieve very high intakes of natural folates through diet alone. As supplemental levels of natural folates can be provided in the form of 5MTHF, it is important to investigate the cellular and nutritional effects of this supplemental form to aid in clinical and public health recommendations.

This thesis aimed to compare the effects of FA vs. 5MTHF supplementation on intracellular folate metabolism and one-carbon transfer reactions in human colon adenocarcinoma cell lines, HCT116 and Caco-2. Cellular proliferation, intracellular folate concentrations, mRNA expression of key genes involved in folate metabolism and one-carbon transfer reactions, and global DNA methylation were investigated. It is anticipated that this study will clarify the impact of equimolar doses of FA and 5MTHF on cellular metabolism which will facilitate better clinical decision making and public policy on folate supplementation and fortification to improve human health while avoiding undue risks.
Chapter 2  Literature review

2.1  Folate

Folate is a water-soluble B vitamin that is involved in nucleotide biosynthesis, the methionine cycle, serine and glycine biosynthesis, and biological methylation reactions (7, 8). Dietary sources include green leafy vegetables, asparagus, broccoli, brussels sprouts, citrus fruit, legumes, organ meats, and folic acid fortified foods (8). Folate occurs naturally in reduced forms and synthetically in the oxidized form, FA. Intestinal microbiota are capable of de novo synthesis of folate in varying amounts depending on the bacterial species (9, 10). The enterohepatic cycle is also involved in the reabsorption of 5MTHF (9, 10) and is a pivotal component of folate homeostasis (11).

2.1.1  Chemistry (folate vs folic acid):

Folate is composed of three moieties: a pteridine ring, para-aminobenzoic acid (PABA) and a polyglutamic acid chain (up to 9 residues) ((12); refer to Figure 2.1). Naturally occurring folates generally have reduced pteridine rings, and are susceptible to oxidative chemical rearrangements and, consequently, loss of activity (13). In contrast, FA, not found in nature, is a synthetic form of folate with a fully oxidized pterine ring and only one glutamate residue, thereby increasing its bioavailability and stability in light and food processing (14). Due its low cost and stability, FA is used in fortified foods and supplements (15). Before FA can be incorporated into the folate pathway and used for cellular processes, it must be reduced to dihydrofolate (DHF) then tetrahydrofolate (THF) and then it may be methylated (12, 15). 5MTHF is a naturally occurring folate and can enter the folate pathway freely without being further reduced (12). When FA and natural folates are consumed, cells (the enterocytes and to a larger degree, the liver) metabolize them to 5MTHF, which makes up 98% of the circulating
folate in the blood (3) and is the only folate form able to cross the blood brain barrier (16). 5MTHF is also known as L-5-MTHF, (6S)-5-MTHF, and levomefolate. It has become commercially available in supplements as a 5MTHF Ca+ salt (Mefolín®, Merk Eprova AG, Switzerland). A distinction between the two 5MTHF enantiomers exists: L-5MTHF is the active form of folate whereas D-5MTHF is the inactive form. Previously, companies supplied 5MTHF with both diasterioisomers because of instability. Now, the Ca+ salt allows the active form to be much more stable with a longer shelf life (17).

Figure 2.1 Chemical structure of folic acid and 5MTHF. Folic acid is shown at the top with all pteridine rings oxidized. The bottom shows the structure of reduced folates and positions of one carbon substitutions. In nature, 5MTHF is polyglutamylated but synthetic 5MTHF-Ca is monoglutamated. Adapted and reprinted by permission from the publisher (Elsevier) (24). Copyright © 2010 Elsevier
2.1.2 *Intake of Folate:*

Mammals are generally unable to synthesize folate as they lack the enzyme which couples the pteridine ring to PABA (8; Figure 2.1). Intestinal microbiota can provide monoglutamylated folates through *de novo* synthesis (9, 10), which is absorbed in the colon (18). For humans, however, folate requirements must be met largely by dietary or supplemental sources (9,10). Due to the variable bioavailability and stability of naturally occurring folates, intake is defined using dietary folate equivalents (DFE). One DFE is equal to 1µg of naturally occurring dietary folate, 0.6 µg of FA when taken with food, or 0.5 µg of FA on an empty stomach (1). Below is a list of the recommended dietary intake (RDA) of folate in North America. The upper tolerable intake (UL) of 1 mg/day for men and women applies to FA only. It was set to prevent the masking of B12 deficiency (1). There is currently no UL for natural folates in North America as it is not possible to consume high levels of natural folates through diet alone (1).

**Table 2.1 Recommended Dietary intake of folate** (1)

<table>
<thead>
<tr>
<th>Age</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults and children ages 14+</td>
<td>400 ug DFE</td>
<td>400 ug DFEs</td>
</tr>
<tr>
<td></td>
<td>600 ug DFE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(pregnancy)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500 ug DFE (lactation)</td>
<td></td>
</tr>
<tr>
<td>Children and infants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth to 6 months</td>
<td>65 ug DFE</td>
<td>65 ug DFE</td>
</tr>
<tr>
<td>7-12 months</td>
<td>80 ug DFE</td>
<td>80 ug DFE</td>
</tr>
<tr>
<td>1-3 years</td>
<td>150 ug DFE</td>
<td>150 ug DFE</td>
</tr>
<tr>
<td>4-8 years</td>
<td>200 ug DFE</td>
<td>200 ug DFE</td>
</tr>
<tr>
<td>9-13 years</td>
<td>300 ug DFE</td>
<td>300 ug DFE</td>
</tr>
<tr>
<td>Upper Limit (UL)</td>
<td>For FA only: &gt;1000 mg/day</td>
<td></td>
</tr>
</tbody>
</table>
2.1.3  Biomarkers of folate status

Folate status can be determined by serum and RBC folate concentrations as well as homocysteine concentrations (19). Serum folate concentrations reflect short-term dietary intake and RBC folate concentrations reflect long-term status and is considered more reflective of folate tissue stores (1). RBC folate is directly reflective of bone marrow folate stores at the time of erythropoiesis and the 120-day turnover rate of RBC makes this measure resistant to short-term folate variation (1). RBC folate concentrations are used to diagnose clinical folate deficiency. According to WHO standards, RBC folate concentrations of <340 nM or serum folate concentrations of <10 nM observed repeatedly over a 1-month period are indicative of folate deficiency (20). There are currently no established high cut-offs for RBC and serum folate concentrations. The 97th percentile of RBC folate concentrations (1360 nM) in the 1994-2004 National Health and Nutrition Examination Survey (NHANES) has been used as an arbitrary cut-off by Colapinto et al. (4).

Homocysteine is a nonspecific inverse indicator of folate status and at concentrations above 16 µM can indicate folate deficiency but lower levels have been used (1). Folate, in the form of 5MTHF, is required to remethylate homocysteine to methionine. During folate deficiency, there is reduced conversion of homocysteine to methionine, thereby increasing homocysteine concentrations (1). Raised homocysteine concentrations can also indicate inadequate vitamin B12 and vitamin B6 status (1). Renal dysfunction and aging can also raise homocysteine concentrations (1).
2.1.4 Folate absorption and metabolism (refer to figure 2.2)

Figure 2.2 Simplified intracellular folate metabolism and 1-carbon transfer reactions in colonic epithelial cells, highlighting the genes that are involved in intraluminal folate hydrolysis (GCPII, Glutamate carboxypeptidase II), intracellular folate uptake (FR-α, Folate receptors; RFC, Reduced folate carrier), intracellular folate retention (FPGS, Polyglutamate synthase) and hydrolysis and efflux (GGH, γ-glutamyl hydrolase), methionine cycle (MTR, Methionine synthase; MTRR Methionine synthase reductase; MTHFR, Methylene tetrahydrofolate reductase), maintenance of intracellular folate pool (DHFR, Dihydrofolate reductase; SHMT Serine hydroxymethyltransferase) and nucleotide biosynthesis (TS, Thymidylate synthase), DNA methylation (DNMT1, 3a, 3b, DNA methyltransferase), and DNA demethylation (MBD2, Methyl binding protein 2). CH₃, methyl group; Hcyt, homocysteine; Met, methionine. Filled circle represents a pteridine ring conjugated to para-aminobenzoic acid. Each filled triangle represents a glutamate, which is linked via a gamma peptide bond to form various chain lengths of polyglutamylated folate. Adapted and reprinted by permission from the publisher (Mary Ann Liebert, Inc. Publishers) (39). Copyright © 2012 Mary Ann Liebert, Inc. Publishers

2.1.4.1 Folate absorption and transport

Folates are absorbed in the small intestine, primarily in the acidic cell surface of the duodenum and jejunum (21) (refer to figure 2.2). Before folates can enter the enterocyte, they are
hydrolyzed into their monoglutamate forms by glutamate carboxypeptidase II (GCPII).

Monoglutamylated forms of folate are then transported into the cell by three types of folate carrier, transporter, and receptor: reduced folate carrier (RFC), proton-coupled folate transporter (PCFT), and folate receptors (FR). These transport systems are expressed in tissues in varying ratios. Each of these transport systems have different affinities for different folate derivatives and optimally function at different pH levels (22). RFC mediates cellular uptake of reduced folates and antifolates and is expressed in the small intestine, colon, liver, pancreas, and kidney (23). Its optimum pH is 7.4 (21) and has a saturable uptake at low folate concentrations (22). PCFT functions best at low pH of 5.5 and has a high affinity for oxidized folates as well as reduced folates (21, 24). It is expressed in the small intestine, liver, kidney, colon, spleen, and placenta (5). Finally, FR (FRα, FRβ, and FRγ) are mostly found in the kidney and liver (22). Transport via FR is slow compared to transmembrane carriers (5) (Refer to Table 2.2).
### Table 2.2 Folate transporters and absorption localization, optimal pH, and pharmacokinetics

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Localization</th>
<th>Optimal pH</th>
<th>Pharmacokinetics</th>
</tr>
</thead>
</table>
| Reduced folate carrier (RFC) 46-94 kDa           | Found in almost all human tissues. High expression in placenta and lowest in skeletal muscle | pH dependent (optimum pH = 5) Major route of delivery of folates to systemic tissues at physiological pH | • High affinity for reduced folates ($K_m$ for 5MTHF ~ 2-7µM)  
• Very low affinity for folic acid ($K_i$ ~150 - 200 µM)  
• Solute carrier  
• Anion exchanger  
• Influx $K_m$ comparable at pH7.4 and pH5.5. Fall in transport due to decrease of $V_{max}$ with decreased pH |
| $SLC19A1$ Chromosome 21q22.3                      | Found in proximal and distal colon                |                                                                             |                                                                                 |
| Proton-coupled folate transporter (PCFT)         | Small intestine Kidney Liver Placenta Retina Brain Spleen colon | Major route of absorption at low pH (optimally at 5.5)                      | • Similar affinity for reduced folates and folic acid  
• $K_m$ at apical brush border membrane of jejunum less than 1 µM  
• Solute carrier  
• Folate-proton symporter |
| $SLC46A1$ Chromosome 17q11.2                      |                                                   |                                                                             |                                                                                 |
| Folate receptors (FRα, FRβ, FRγ) 38-40 kDa       | Kidney Liver Choroid plexus Retina Uterus Placenta Human body fluids | Functions best at neutral to mildly acidic pH (5.6 – 7.2)                    | • High affinity for folic acid ($K_d$ 1-10nM)  
• Different affinities for different folate forms  
• FRα binds to 5MTHF better than FRβ.  
• Inefficient transport compared to RFC and PCFT  
• GPI anchored protein  
• Receptor mediated endocytosis  
• Unidirectional |
| $FR$ Chromosome 11q13.2-q13.5                     |                                                   |                                                                             |                                                                                 |
| Glutamate carboxypeptidase II (GCPII) 84-120 kDa  | brain, salivary gland, small intestine lumen, proximal renal tubule, and colonic neuroendocrine cells | Optimal pH: 6.5                                                             | • Transmembrane protein  
• High affinity (nM), low turnover |
| $GCPII$ Chromosome 11p11.2                        |                                                   |                                                                             |                                                                                 |
2.1.4.2 Intracellular folate retention and export (refer to table 2.3)

Once in the enterocyte, monoglutamylated forms of folate are either polyglutamylated by FPGS (folylpolyglutamate synthase) to trap folate inside the cell, or enters the portal hepatic circulation to be distributed to tissues. Polyglutamylated folates are better retained in cells and are better substrates than monoglutamates for intracellular folate-dependent enzymes (23). 5MTHF and FA are poor substrates for FPGS (23). 5MTHF and FA must be metabolized to THF to be effectively polyglutamylated (23). Intracellularly, folates are metabolized to yield 5MTHF, the primary circulating form of folate. Metabolism of folates to 5-MTHF occur as folates pass through the enterocytes; however, metabolism also occurs in the liver (25). In order to leave the cell, polyglutamylated folates must be hydrolysed by gamma-glutamyl hydrolase (GGH) to the monoglutamylated form (26). Folate export is mediated via RFC on the basolateral membrane (15).

### Table 2.3 Intracellular proteins for folate retention, hydrolysis, and export

<table>
<thead>
<tr>
<th>Protein</th>
<th>Localization</th>
<th>Optimal pH</th>
<th>Pharmacokinetics</th>
</tr>
</thead>
</table>
| Polyglutamate synthase (FPGS) 60-70 kDa      | Cancer tissues, Gut, bone marrow stem cells, liver, kidney, intestine, among other tissues | 8.2-8.5    | • Time- and dose- dependent. Higher extracellular folate concentrations result in lower intracellular folate accumulation and retention.  
• Role in folate retention 
• Favours DHF binding and reduced enzymatic activity as glutamate tail increases |
| Gene: FOL3, Chromosome 9q34.11                |                                                        |            |                                                                                   |
| γ-glutamyl hydrolase (GGH) 36-120 kDa        | Lysosome protein found in liver, kidney, serum, placenta, colon, brain, and testes | 4.5-6.0    | • Low affinity (nM), high turnover                                                 |
| GGH, Chromosome 8q12.23-113.1                |                                                        |            |                                                                                   |

2.1.4.3 Intracellular folate metabolism, nucleotide synthesis, and methionine cycle

Folate is important for cell growth and division as they are integrally involved in one-carbon transfer reactions. It is important for the de novo synthesis of thymidylate and purine
nucleotides and for biological methylation reactions including DNA methylation (refer to Table 2.4; also figure 2.2).

2.1.4.3.1 Thymidylate and purine synthesis:

DHFR reduces DHF (a product of converting dUMP to dTMP) to regenerate THF (refer to figure 2.2). THF can re-enter the nucleotide biosynthesis pathway or be used to regenerate 5MTHF via conversion to 5,10-methyleneTHF by Serine hydroxymethyltransferase (SHMT) and then further reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to yield 5MTHF (15).

5MTHF donates a carbon group to the biological methylation pathway (described below), which also yields THF. THF is an important substrate for pyrimidylate and purine biosynthesis, which are the building blocks of DNA and RNA. THF is also a good substrate for FPGS and can be polyglutamylated. SHMT catalyzes the reversible conversion of THF and serine to 5,10-methyleneTHF and glycine (15, 8). 5,10-methyleneTHF transfers a methyl group to deoxyuridine monophosphate (dUMP) to yield dTMP and DHF by thymidylate synthase (TS). dTMP is a precursor of pyrimidylate biosynthesis (27). The products from the previous cycle, THF and 5,10-methyleneTHF also contribute to purine synthesis once they are formylated. THF and serine form 5, 10-methyleneTHF and glycine by SHMT. 5, 10-methyleneTHF can also be converted to 5,10-methylenylTHF and then to 10-formylTHF by MTHFD1 or MTHFD2 (methylene tetrahydrofolate dehydrogenase) (27). In purine synthesis, the carboxyl groups of two 10-formylTHFs are required to yield IMP (inosine monophosphate or inosinic acid), a precursor to the purines AMP (adenosine monophosphate) and GMP (guanosine monophosphate) (27).
2.1.4.3.2 SAM regeneration, methionine cycle, and DNA methylation:

5MTHF is the only form of folate that is able to participate in the regeneration of S-adenosylmethionine (SAM), a universal methyl donor for most biological methylation reactions including DNA methylation (23). 5MTHF provides a methyl group for transmethyltransferation of homocysteine to methionine catalyzed by methionine synthase (MS), a vitamin B12-dependent enzyme (8, 12, 23). Methionine is then converted to SAM by methionine S-adenosyltransferase (MAT) or SAM synthase (25, 28, 29). DNA methylation is mediated by DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) (22). DNMT1 is believed to maintain DNA methylation patterns following DNA replication (28,29) and DNMT 3a and 3b are capable of de novo methylation beyond the inherited patterns (30). Methyl binding protein 2 (MBD2) is important for both as a DNA methylation-dependent repressor and activator of genes silenced by

| Table 2.4 Proteins involved in intracellular folate metabolism, nucleotide biosynthesis |
|----------------------------------------|-----------------|---------------------------------|
| Protein                                | Localization    | Pharmacokinetics                |
| Dihydrofolate reductase (DHFR) 25kDa   | Found abundantly in all tissues | • NADPH dependent enzyme        |
| Gene: DHFR Chromosome 5q14             |                  | • Activity higher in fast growing cells and replicating cells |
| Serine hydroxymethyltransferase (SHMT) 54 kDa |                  | • FA poor substrate for DHFR    |
| Gene: SHMT Chromosome 17p11.2 (cSHMT) and 12q13.2 (mSHMT) |                  | • DHFR protein prevents its own translation in the absence of substrate by binding to its own mRNA |
| Thymidylate synthase (TS) 35 kDa      | Cytoplasm of human cells and tissues | • Affinity for glycine and 5MTHF varies between mSHMT and cSHMT |
| Gene: TS Chromosome 18p11.32           |                  | • Extracellular folate levels may modulate SHMT expression and activity |
|                                        |                  | • Homodimer                     |
|                                        |                  | • TS protein prevents its own translation in absence of substrate by binding to its own mRNA |
|                                        |                  | • 5,10, methyleneTHF competes with TS mRNA and upregulates TS translation |
methylation (31). De novo de-methylation is mediated by ten eleven translocation (TET) 1 protein which oxidizes methylated cytosines (5mC) to 5-hydroxymethylcytosine (5hmC), the first step in DNA de-methylation (81).

Table 2.5 Proteins involved with the methionine cycle and DNA methylation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Localization</th>
<th>Pharmacokinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine synthase (MS) 140 kDa</td>
<td>Almost all tissues, especially in pancreas</td>
<td>• NADPH and cobalamin dependent</td>
</tr>
<tr>
<td>Gene: MTR</td>
<td></td>
<td>• Oxidizes Cob(II)alamin to Cob(I)alamin</td>
</tr>
<tr>
<td>Chromosome 1q42.3-1q44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine synthase reductase (MTRR) 78 kDa</td>
<td>All Tissues</td>
<td>• NADPH and cobalamin dependent</td>
</tr>
<tr>
<td>Gene: MTRR</td>
<td></td>
<td>• Reduces Cob(I)alamin to Cob(II)alamin</td>
</tr>
<tr>
<td>Chromosome 5p14.2-15.3</td>
<td></td>
<td>• Reduces MS</td>
</tr>
<tr>
<td>Methylene tetrahydrofolate reductase (MTHFR)</td>
<td>All Tissues</td>
<td>• NADH or NADPH dependent</td>
</tr>
<tr>
<td>74.5 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene MTHFR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromosome 1p35.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl binding protein 2 (MBD2) 49 kDa</td>
<td>Downregulated in cancers</td>
<td>• DNA demethylation</td>
</tr>
<tr>
<td>Gene MBD2</td>
<td></td>
<td>• Favours fully methylated DNA to hemi-methylated DNA</td>
</tr>
<tr>
<td>Chromosome 18q21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA methyl transferase (DNMT1) 150-190 kDa</td>
<td>Normal human tissues, fetal tissues, and some tumor tissues. Highest mRNA</td>
<td>• DNA methylation maintenance</td>
</tr>
<tr>
<td>Gene: DNMT-1</td>
<td>expression in heart, brain, and tumour tissues</td>
<td>• Favours hemi-methylated cytosines as substrates</td>
</tr>
<tr>
<td>Chromosome 19p13.2-3</td>
<td></td>
<td>• Regulated post-transcriptionally and by cell cycle</td>
</tr>
<tr>
<td>DNAMT 3a and 3b</td>
<td>Found in majority of human cells</td>
<td>• De novo DNA methylation</td>
</tr>
<tr>
<td>Gene: DNMT-3a</td>
<td></td>
<td>• DNA methylation maintenance</td>
</tr>
<tr>
<td>Chromosome 2p23.3</td>
<td></td>
<td>• Prefers hemi-methylated cytosines to non-methylated</td>
</tr>
<tr>
<td>Gene: DNMT-3b</td>
<td></td>
<td>• methylated cytosines</td>
</tr>
<tr>
<td>Chromosome 20q11.2</td>
<td></td>
<td>• May be regulated by de novo DNA methylation</td>
</tr>
</tbody>
</table>

2.2 Folate and Health

Folate plays an important role in human health and disease as it is essential for nucleotide biosynthesis and biological methylation reactions. Folate deficiency has been associated with
many diseases such as megaloblastic anemia, cardiovascular disease, cognitive impairments, NTDs, and cancer (15). Folate deficiency can occur due to insufficient dietary intake, impaired folate absorption and metabolism, and/or increased demand and utilization (1, 15). Some of the well-known contributors to folate deficiency include: poor-quality diet or lack of folate in diet (from overcooked food or lack of fruits and vegetables), gastrointestinal disorders impairing folate absorption such as celiac disease and Crohn’s disease, long term alcoholism, tobacco smoking, medical conditions that increase rates of cell turnover such as inflammatory bowel disease and pregnancy, which in turn raise folate requirements, and drugs such as antifolates used in chemotherapy as well as certain anti-epileptic and anti-inflammatory medications that interfere with folate absorption and/or metabolism (1, 8, 15, 31).

FA supplementation has been used to prevent and treat some of the aforementioned conditions associated with folate deficiency or insufficiency. FA supplementation is highly effective and successful in treating megaloblastic anemia (23, 32) and especially preventing NTDs (33-35). FA supplementation has been generally regarded a safe and has long been presumed to be purely beneficial (36). More recently, however, FA supplementation has also been linked to adverse health outcomes including cancer promotion, decreased natural killer cell cytotoxicity, potential for development of tolerance or resistance to antifolate drugs used in treatment of cancer and inflammatory conditions, accelerated cognitive impairment in conjunction with low vitamin B12 status, and aberrant or dysregulation of DNA methylation (7, 15, 37).

The evidence regarding the role of FA supplementation in health and disease is still inconsistent but it was concluded by an expert panel commissioned by the U.S. National Toxicology Program and the Office of Dietary Supplements of the National Institutes of Health
that there is no benefit of supplemental folate in reducing cancer with adequate baseline folate status (38). They also determined that there is enough consistent evidence from human studies of adverse effect on cancer growth from supplemental FA to justify further research (38) Some relationships have been well elucidated and others less so. Warszyszynska and Kim summarized the strength of evidence concerning the effects of FA supplementation on health and disease (15, see Table 2.6). The exact nature and magnitude of the effects of FA supplementation on health outcomes still remain to be elucidated.

Table 2.6 Strength of evidence for effects of FA on health and disease

<table>
<thead>
<tr>
<th>Evidence</th>
<th>Effects</th>
<th>Null</th>
<th>Adverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convincing</td>
<td>* Macrocytic anemia</td>
<td>* Coronary heart disease</td>
<td>* Masking vitamin B₁₂ deficiency</td>
</tr>
<tr>
<td></td>
<td>* Neural tube defects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probable</td>
<td>* Prevention of new cancer</td>
<td>* Other congenital defects</td>
<td>* Promotion of (pre)neoplastic lesions</td>
</tr>
<tr>
<td></td>
<td>* Stroke</td>
<td></td>
<td>* Epigenetic Δ</td>
</tr>
<tr>
<td></td>
<td>* Cognitive function</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Congenital heart defects</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Epigenetic Δ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible/</td>
<td>* Pregnancy outcomes</td>
<td>* Resistance to antifolates</td>
<td></td>
</tr>
<tr>
<td>insufficient</td>
<td>* Neuropsychiatric disorders</td>
<td>* Accelerated cognitive dysfunction with</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>low vitamin B₁₂ status</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>* ↓ NK cell activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>* ↑ risk of obesity, insulin resistance in</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>offspring</td>
<td></td>
</tr>
</tbody>
</table>

*Note: Δ, Alterations; ↑, Increased; ↓, Decreased*

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2.2.1 Folate and anemia

Folate deficiency leads to megaloblastic anemia (24). Megabloblastic anemia is characterized by enlarged nucleated erythrocyte precursor cells with noncondensed chromatin due to defective DNA synthesis and hypersegmented neutrophils (37). In later stages macrocytic anemia, hematocrit and hemoglobin decrease occur (37). Symptoms include weakness, fatigue, irritability, difficulty concentrating, and shortness of breath (1, 39). FA supplementation has been shown to be an effective treatment in folate-deficiency-associated megaloblastic anemia (2, 24).
2.2.2  Folate and masking of B12 deficiency

The tolerable UL for FA (1 mg/day) was set to prevent masking of B12 deficiency (1). B12 deficiency can result in cognitive and neuropsychological disorders and can also cause megaloblastic anemia (1). However, high levels of FA can mask the characteristic megaloblastic anemia associated with B12 deficiency that would alert clinicians to investigate vitamin B12 deficiency. As a result, the neurological damage may go initially undetected and progress (1, 40, 41). Proposed mechanisms suggest that an abundance of FA allows for the continuous production of nucleotides for DNA synthesis (24). However, SAM regeneration (integral to the protein and DNA methylation pathways) is dependent on B12 as a cofactor for MS and cannot occur during B12 deficiency (1).

Masking B12 deficiency is most common in those with low B12 status. It was previously believed that the elderly were more susceptible to B12 deficiency because 10-30% of older adults develop gastric atrophy, a condition which decreases secretion of hydrochloric acid in the stomach and subsequent decrease in B12 absorption (1, 40). The Institute of Medicine (IOM) recommended that adults over 50 years achieve their B12 intake through supplements or fortified foods (1). Due to this recommendation, the elderly are more likely to be exposed to higher FA intakes from supplements and from their diets, which are generally high in grain products (5, 15). It has been found more recently that rates of B12 deficiency is uniform among the Canadian population (40).

2.2.3  Folate and cardiovascular disease

Elevated homocysteine concentrations have been associated with an increased risk of coronary heart disease and stroke (42). Furthermore, low folate status has been associated as a risk factor for coronary heart disease independent of homocysteine levels (42). The
Homocysteine Studies Collaboration, which examined data from 30 prospective and retrospective studies, reported an 11\% of coronary heart disease and a 19\% decreased risk of stroke for every 25\% reduction in homocysteine concentrations (corresponding to approximately 3 µM) (15, 43). Due to folate’s role in converting homocysteine to methionine and the observations that homocysteine levels are readily reduced by FA supplementation (44, 45), it has been suggested that FA supplementation may be protective against cardiovascular disease. While observational epidemiologic studies have generally been supportive of a protective effect of folate and FA on coronary heart disease and stroke (42), randomized clinical trials utilizing FA supplementation, either alone or in combination with other B vitamins, conducted in highly predisposed individuals with previous coronary events have generally reported a null effect on the secondary prevention of coronary heart disease (15, 42, 43, 46, 47). Although epidemiological and randomized intervention clinical trial data provide stronger support for a protective effect of FA supplementation on stroke prevention compared to coronary heart disease, the data are inconsistent and equivocal (42, 43, 47, 49).

2.2.4 Folate and neuropsychiatric disorders

Folate deficiency, especially if left untreated, is often associated with depression and cognitive decline (48). With FA supplementation, patients generally improve (49). Neurological complications such as developmental delay and cognitive impairment are also observed in patients with inborn errors of folate transport and metabolism (49). However, the effect of mild to moderate folate deficiency or insufficiency on cognitive impairment and dementia is equivocal (49). Hyperhomocysteinemia and low folate status have been suggested to be independent risk factors for dementia by several observational epidemiological studies (49). Proposed mechanisms behind the observed association between folate deficiency and neurological
disorders involve reduction in availability of SAM required for methylation processes (49) and vascular and neurotoxic mechanisms mediated by hyperhomocysteinemia such as oxidative stress and impairment of glutathione metabolism (48, 49). Although not uniformly consistent, randomized clinical trials of FA supplementation, either alone or in combination with other B vitamins, in predisposed individuals have generally reported an improvement of cognitive function when supplementation is provided over long durations (49). Interestingly, a study by Morris et al. found that seniors with low B12 status and high serum folate were associated with cognitive impairment (40). However, at normal B12 status, high serum folate was protective against cognitive impairment (40).

With respect to folate and depression, supplementation of folate, regardless of folate type (FA or 5MTHF) has been associated with general improvements in mood and morbidity (50-55). Papakostas et al. found that 15 mg of 5MTHF for 60 days significantly improved mood in selective serotonin reuptake inhibitor (SSRI) resistant patients with major depressive disorder (55). Preliminary evidence of 5MTHF monotherapy having an antidepressant effect in the absence of folate deficiency has been reported (53); however, this effect was most observed in patients whose RBC folate concentrations increased with treatment in the absence of folate deficiency (49, 53, 56). A proposed mechanism for this mood improvement is related to that of nitrous oxide (laughing gas) on creating euphoria (49). Nitrous oxide inactivates MS and results in increased levels of circulating 5MTHF, which would increase levels of 5MTHF in the nervous system and mimic the euphoria induced by nitrous oxide (49). Another proposed mechanism postulates that increasing folate availability will increase the levels of SAM, allowing for methylation of myelin basic protein and membrane phospholipids to maintain myelination in the nervous system. Neuropsychiatric disorders are associated with lower levels of SAM and thus
lower methylation activity (15, 49, 55). In patients with major depressive disorders, folate deficiency has been linked to reduced response to antidepressant treatment (48).

2.2.5 Folate and cancer

Epidemiologic studies have suggested that folate insufficiency may increase the risk of several human cancers and that FA supplementation may reduce this risk (57). A large body of evidence from animal studies and clinical trials, however, suggests that folate may possess dual modulatory effects on colorectal cancer development and progression depending on the dose and the stage of cell transformation at the time of folate intervention (12, 15, 28, 36) (refer to figure 2.3). Collectively, animal studies demonstrate that folate deficiency has an inhibitory effect whereas FA supplementation has a promoting effect on the progression of established (pre)neoplastic colorectal lesions (8, 59-61). In contrast, in normal colorectal mucosa, folate deficiency predisposes it to neoplastic transformation, and modest levels of FA supplementation suppress, whereas supraphysiologic supplemental doses enhance, the development of cancer in normal colorectal mucosa (8).

The potential tumor promoting effect of high FA was further corroborated by the Aspirin/Folate Polyp Prevention Study, which reported that FA supplementation at 1 mg per day for 6 years in individuals likely harboring (pre)neoplastic lesions significantly increased the risk of colorectal cancer (62 and prostate cancer (63). However, a combined analysis of 3 large randomized clinical trials of FA supplementation for the prevention of colorectal cancer reported a null effect (63). Combined or meta-analyses of several large randomized clinical trials that investigated the effect of FA supplementation with or without other B vitamins on cardiovascular disease outcomes as the primary endpoint reported either a tumor promoting (64) or null (43) effect on cancer risk as the secondary endpoint. Two most recent systemic review and meta-
analysis of FA trials with cancer risk as either the primary or secondary endpoint again reported either an increased (65) or null (66) effect associated with FA supplementation. Two ecologic studies that examined a temporal post-fortification trend of colorectal cancer incidence in the US, Canada and Chile reported increased colorectal cancer rates in these countries following fortification, suggesting that FA fortification may have been wholly or partly responsible for this trend (67, 68). However, two recent large population-based cohort studies have reported a decreasing trend of colorectal cancer incidence post-fortification in the US (69, 70).

Several potential mechanisms relating to the role of folate in one-carbon transfer reactions and consequent DNA synthesis and epigenetic regulations exist to explain the dual effects of folate on cancer development and progression. The first is folate’s important role in DNA synthesis, integrity, and repair (8, 39). In normal tissues, an abundance of folate ensures the fidelity of DNA replication and stability (8, 71). However, folate deficiency leads to DNA strand breaks, chromosomal and genomic instability, uracil misincorporation, increased mutations, and impaired DNA repair (8, 74-76). FA supplementation can prevent and correct some of these defects (8, 74-76). In contrast, in (pre)neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, folate deficiency causes ineffective DNA synthesis, resulting in inhibition of tumor growth and progression (8, 76). The most likely mechanism by which FA supplementation may promote the progression of established (pre)neoplastic lesions is the provision of nucleotide precursors to rapidly replicating cells for accelerated proliferation and cancer progression (15).

The second mechanism related to folate’s role in DNA methylation (39) as discussed in the next section. However, the effects of folate on DNA methylation has not yet been well established (8). Folate, in the form of 5MTHF, is involved in remethylation of homocysteine to
methionine, which is a precursor of SAM, the primary methyl group donor for most biological reactions including DNA methylation (15) (see figure 2.1). Aberrant DNA methylation due to variation in folate status can contribute to carcinogenesis via global DNA hypomethylation, which is associated with genomic instability, via DNA hypermethylation at promotor and/or regulatory regions of tumor suppressor and mismatch repair genes with consequent gene silencing, and via hypermutability of methylated cytosine (39). In normal cells, folate deficiency may cause global DNA hypomethylation with consequent genomic instability, leading to neoplastic transformation (36, 39). In transformed cells, however, folate deficiency may suppress tumor progression by reversing promotor cytosine-guanine (CpG) island DNA methylation of tumor suppressor and other cancer related genes; this leads to reactivation of these epigenetically silenced genes (39). In normal cells, FA supplementation may prevent global DNA hypomethylation, thereby reducing the risk of neoplastic transformation by ensuring genomic stability (39). In transformed cells, however, FA supplementation may promote tumor progression by inducing de novo methylation of promotor CpG island of tumor suppressor and cancer related genes, thereby silencing these genes (39). However, the effects of folate deficiency and FA supplementation on DNA methylation are highly complex as they are 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 (36, 39).
Figure 2.3 The Dual Modulatory Role of Folate in Carcinogenesis: cancer develops through stages of premalignant lesions in the target organ. In normal tissues, folate deficiency or folate supplementation at supraphysiological doses enhances the development of preneoplastic lesions and development of tumours while modest supplemental levels protect against lesion development. In tissues with established neoplasms, folate supplementation has a cancer promoting effect while folate deficiency provides a cancer inhibitory effect. Folate’s dual modulatory effect is dependent on the dose and timing of folate intervention due to its key role in DNA synthesis and repair, biological methylation reactions, and DNA methylation reactions. Reproduced with permission from the publisher John Wiley and Sons Ltd. (15). Copyright © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

2.2.6 Folate and epigenetics

Epigenetics is defined as the inheritance of information regarding gene expression levels mediated by mechanisms other than DNA sequence changes including histone modifications, chromatin remodelling, DNA methylation and RNA interference (39). In contrast to genetics, epigenetic changes are gradual in onset and progressive. Furthermore, the effects are dose dependent and reversible and modifiable by environmental factors (39, 77).

One of the main epigenetic DNA modifications in mammals is the methylation of cytosine located within the CpG sequences, which is heritable, and tissue- and species-specific (39). DNA methylation is an important epigenetic determinant in gene expression (an inverse
relation except for few exceptions), in the maintenance of DNA integrity and stability, in chromatin modifications, and in the development of mutations (39). Up to 80% of all CpG sites in human DNA are normally methylated (78). However, this methylation occurs primarily in the bulk of the genome where CpG density is low, including exons, noncoding regions, and repeat DNA sites, and allows correct organization of chromatin in active and inactive states (39, 79). By contrast, most CpG rich areas clustered in small stretches of DNA termed “CpG islands”, which span the 5’ end of approximately half of the human genes including the promoter, untranslated region, and exon 1, are unmethylated in normal cells, thereby allowing transcription (78). When methylated, CpG islands cause stable heritable transcriptional silencing (78), which is mediated by the transcriptional repressor, methyl-CpG binding protein-2 (MeCP2), which binds methylated CpG islands and recruits a complex containing a transcriptional co-repressor and a histone deacetylase (HDAC) (39). Deacetylation of histones suppresses transcription by allowing tighter nucleosomal packaging and thus rendering an inactive chromatin conformation (39).

DNA methylation is a dynamic process between active methylation, mediated by CpG methyltransferases (DNMT1, 3a, 3b) (39, 80) using SAM (39, 80), and removal of methyl groups from 5-methylcytosine (5-mC) residues by several mechanisms (39, 80), including active demethylation by a purported demethylase, methyl DNA-binding domain protein 2 (MBD2) (39, 80). Most recently, hydroxymethylcytosine (5-hmC) was found and proposed as the initial demethylation step. The conversion of 5-mC to 5-hmC in mammalian DNA is mediated by the methylcytosine oxygenase TET1 (ten-eleven translocation 1) (81).

Aberrant patterns and dysregulation of DNA methylation are mechanistically related to the development of several human diseases including cancer (69). Of all epigenetic modifications, DNA methylation has garnered most interest in the field of folate and epigenetics.
As a methyl donor critically involved in the provision of SAM, folate status has been shown to modulate DNA methylation especially in two important life stages: pre-/early postnatal life and aging (39).

*In vitro* studies conducted in human cancer cell lines collectively suggest that effects of folate deficiency and FA supplementation on DNA methylation are cell, site and gene-specific and that the direction of DNA methylation changes may not be the same between global and gene or site-specific DNA methylation (36, 39, 59). The major limitation of the *in vitro* system to study the effect of folate on DNA methylation is that the degree of folate deficiency and FA supplementation used in this system is not physiologically and clinically relevant and applicable to *in vivo* situations. Further, many of these systems have predominantly investigated the effects of FA alone while circulating forms include both 5MTHF and FA. Nevertheless, these *in vitro* studies collectively provide proof-of-principle that supports the ability of folate to modulate DNA methylation.

The available evidence from animal studies indicates that the effects of folate deficiency and FA supplementation on DNA methylation are highly complex as they are gene and site-specific and depends on species, cell type, target organ, and stage of transformation as well as on the timing, degree and duration of folate manipulation (39). Data from these animal studies may not be entirely applicable to humans as there are significant inherent differences in folate metabolism and epigenetics between animal models and humans. Nevertheless, these studies provide experimental evidence to support the ability of isolated folate deficiency or FA supplementation alone to modulate global and gene-specific DNA methylation.

Although not uniformly consistent, the data from human studies render stronger support for the folate’s ability to modulate global DNA methylation in peripheral blood mononuclear
cells (PBMC) and in the colorectum, the target tissue of interest (39). Most of these studies indicate a positive correlation between global DNA methylation in PBMC and in normal-appearing colorectal mucosa (39). Generally, folate deficiency appears to induce PBMC global DNA hypomethylation but there is no conclusive data suggesting that folate deficiency of a physiologically and clinical relevant degree induces significant global DNA hypomethylation in the colorectum (39). In contrast, FA supplementation, even at the modest supplemental levels, appears to be able to increase global DNA methylation in normal-appearing colorectal mucosa in certain situations (39). Overall, FA supplementation, even at modest supplemental levels, appears to be able to reverse pre-existing global DNA hypomethylation and to increase the extent of global DNA methylation above the pre-existing level in PBMC and the colorectum. The effect of folate on gene-specific DNA methylation is not well established. Most of these studies used a candidate gene approach to interrogate a limited number of genes. A comprehensive survey of the effect of folate status on gene and site-specific DNA methylation using an epigenomic approach is warranted.

Emerging evidence suggest that DNA methylation patterns of a developing fetus can be modulated by environmental intrauterine exposures including folate and FA. Evidence from Waterland and Jirtle (82) using the yellow *agouti* mice demonstrated that maternal supplements containing FA and other B vitamins can increase CpG methylation in the promoter region of the *agouti* gene. When the promotor region is methylated, the obese and yellow colour phenotypes of the pups are permanently changed to brown and thin. Another study by Waterland et al (83) observed the effect of methyl donor supplementation on *Axin(Fu)*+ offspring. Pups born to mothers supplemented with methyl donor supplementation before and during pregnancy had increased DNA methylation of the *Axin(Fu)* gene and reduced tail kinking. As discussed in
sections 2.2.7 and 2.2.8 below, excess maternal folate intake and/or FA supplementation above the beneficial levels may negatively impact fetal growth and development and subsequent risk for disease later in life in part via change in DNA methylation of the developing fetus (39).

2.2.7 Folate and pregnancy

During pregnancy, folate requirements increase. Uterine, placental, and foetal growth results in greater need for one-carbon transfer reactions involved with DNA synthesis and cell replication and thus a higher requirement of folate (1). Folate receptors are highly expressed in the placenta and folate is actively transported to the fetus against a concentration gradient, resulting in higher cord blood folate concentrations compared with maternal blood folate concentrations (84). Folate deficiency during pregnancy can result in maternal megaloblastic anaemia. It is estimated that 35%-75% of pregnant women in developing countries develop anemia (16). While the most common cause of anemia during pregnancy is iron deficiency, folate intake that does not meet the increased requirements during pregnancy may result in maternal folate deficiency and manifest as megaloblastic anemia. Therefore, FA supplementation is recommended throughout pregnancy and not only during the periconceptional period (84).

In addition to the associations between folate deficiency during pregnancy and the risk of congenital defects (discussed in the next section), folate deficiency has also been associated with adverse pregnancy outcomes such as placental abruption, preeclampsia, spontaneous abortion and still birth, although supporting evidence has been equivocal (84, 85). The effects of folate status on birth weight have been documented in women in countries and communities with low maternal dietary folate (85). Generally, low maternal folate status and intake are associated with an increased risk of low infant birth weight or premature infant delivery (85, 86-89).
Excess folate during pregnancy has been associated with negative health outcomes in offspring such as increased rates of twinning and miscarriage, although this remains unproven (84). Furthermore, while other studies have not found an association between excess folate and negative health outcomes in offspring (15), recent studies have reported an increased risk of asthma, allergies, and autism in the offspring associated with maternal FA supplementation (15, 90). The Pune Study in India has reported that high folate and low B12 status during pregnancy can lead to increased adiposity and greater risk for insulin resistance of offspring (7). In rodent studies, effects of maternal folic acid supplementation included increased mammary tumor risk in female offspring (9), lower body weight and reduced femoral area and strength after weaning in female offspring (91), increased glucose concentration regardless of folate level during weaning (92), and increased insulin resistance in male offspring (93). An interesting study by Cho et al (94) found that male pups weaned from dams fed with 10-fold FA diet during pregnancy responded differently depending on their post-weaning diet. When male pups were continued on the high vitamin diet, they showed reduced obesogenic behaviour and physiological response. When male pups were fed the recommended vitamin diet, they showed increased obesogenic behaviour and physiological response (94). These observations illustrate the incredibly complex role of maternal folate intake and status on offspring health outcomes.

2.2.8 Folate and fetal development

Folate is vital for fetal development. While it is unclear what the underlying mechanisms of the different congenital defects are, folate plays a key role during the periconceptional period of pregnancy as it is important for proper closure of the neural tube. Two common types of NTDs include spina bifida and anencephaly and are most commonly seen associated with sub-optimal folate status (95). The Leeds Pregnancy Nutrition study first made the association
between folate status and NTDs (15). Following this observation, two large randomised double-blind clinical trials successfully demonstrated reduced NTD risk with periconceptional FA supplementation (85). Since then, many subsequent studies demonstrated the effectiveness of maternal FA supplementation on reducing NTDs (15). Unfortunately, the association between FA supplementation and other congenital defects have not been clearly defined. However, few studies have shown the protective effect of FA supplementation on congenital heart defects, urinary tract anomalies, and limb defects (15, 85).

2.3  Folic Acid: fortification, intakes, and potential cellular outcomes

Mandated FA fortification, discretionary FA supplementation, and prevalent supplemental use, especially in pregnant women and cancer patients, have increased the intake and blood levels of folate in North America (3). Although FA fortification has achieved its primary objective, that is decreasing the rate of NTDs, and FA supplementation may provide several health benefits, albeit unproven, an emerging body of evidence suggests that high folate status, primarily from high FA intake or high levels of UMFA, may be associated with adverse health outcomes (8, 71). In this section, I will discuss FA fortification and supplementation, FA metabolism, the metabolic outcomes of high FA intake, and arguments for 5MTHF supplementation.

2.3.1  Folic acid fortification and supplementation

Mandatory FA fortification has been implemented in many countries as a public health measure to reduce the risk of NTDs. In 1998, Canada mandated FA be added to all white flour and enriched pasta and cornmeal products sold in Canada (96, 97). In Canada, all white wheat flour and cornmeal are fortified at a minimum level of 150 µg/100 g and enriched pasta at a level of 200 µg/100 g (96). In the US, all white wheat flour, corn meal, and enriched pasta are fortified
at a minimum level of 140 µg/100g (97). In Canada, FA fortification has been successful in reducing overall rates of NTD by 50% (85). Due to the increased folate requirement during pregnancy, pregnant and lactating women are recommended to take a multivitamin with 400 µg of folic acid in addition to dietary folate by a number of expert bodies including, Society of Obstetricians and Gynecologists of Canada, and Canadian task force on the Periodic Health Examination (98). However, most prenatal vitamins available in Canada contain 1 mg FA. The Public Health Agency of Canada’s 2006 Maternity Experiences Survey reported that 58% of Canadian women took multivitamins containing FA in the 3 months prior to pregnancy (99). A cohort of pregnant women from Toronto reported that overall, 60% of women reported using a B vitamin containing supplement before pregnancy and around 90% of women reported taking a B vitamin containing supplement during pregnancy (100). In this study, the median supplemental intake of FA was 1,000 µg per day. Comparatively in the US, data from the NHANES survey showed that 77% of pregnant women reported use of supplements and almost 90% of women reported use of FA containing supplement by their third trimester (101). The mean FA supplementation was 817 µg/day.

It is estimated that the Canadian population consumes 200 µg/day from natural dietary sources and 100-200 µg FA/day from fortified sources in the post-fortification era (3), thereby potentially exceeding the RDA. Standard multivitamins typically contain 400 µg of FA. Approximately 40% of the North American population consumes FA containing supplements and use of dietary supplements is associated with the female gender, older age, higher socioeconomic status, and a healthy lifestyle (102). Multivitamin supplement use is also common in cancer patients and survivors (103). In the US, approximately 50% of adults or children who regularly consume supplements exceed the UL for FA (1 mg/day) (3).
Research by Shakur et al. has identified discrepancies in the actual amount of FA in foods and what is stated in the label (104). In order to ensure the product remains above the mandated fortification level, manufactures often add overages of FA. In the US, FA levels twice the mandated minimum have been reported (105). Until recently there has been no analysis of FA fortified food. This raises the concern that individuals may unintentionally consume FA at levels above the UL, especially if taking supplements. The USDA’s Dietary Supplement Ingredient Database also reports nutrient overages in dietary supplements (105).

Blood levels of folate and FA have significantly increased in the post-fortification era in North America (4, 106). Data from the NHANES 1988–2010 in the US revealed 2.5X and 1.5X increased geometric mean concentrations of serum and RBC folate, respectively, in the post-fortification era (1999–2010) relative to pre-fortification (1988–1994) concentrations (106). Data from the Canadian Health Measures Survey (CHMS) 2007–2009 revealed a virtual non-existence of folate deficiency (defined by IOM, 1998 standards as RBC folate concentrations <305 nmol/L) in the Canadian population, whereas > 40% of the population had high RBC folate concentrations (defined as levels above the 97th percentile of RBC folate concentrations from NHANES 1999–2004 at 1360 nmol/L) (4). RBC folate concentrations greater than 906 nM (4) have been shown to be associated with protection against NTDs. In the aforementioned Canadian study, approximately 25% of women at reproductive age had RBC folate concentrations below this cut-off associated with NTD protection (4).

The detection of circulating UMFA has also increased in the North American population post-fortification (15, 107). In the Framingham Offspring Cohort study, the prevalence of detectable UMFA was 75% post-fortification in comparison to 55% pre-fortification in supplement nonusers and 81% post-fortification in comparison to a pre-fortification prevalence
of 72% in supplement users (107). Data from NHANES 2001–2002 revealed detectable UMFA in 38% of the US population aged ≥60 years (26). A study examining postmenopausal women in the post-fortification era reported a 78% prevalence of detectable UMFA (108). In addition, the detection of UMFA is not limited to countries with mandatory FA fortification; UMFA was detected in a majority of samples in a study conducted in Ireland where only voluntary FA fortification takes place (109). The role of UMFA in human health and diseases has not been clearly elucidated and is hotly debated. Some have suggested that UMFA might be primarily responsible for the purported adverse effects of excessive FA intake (15), although no convincing scientific evidence exists to support this conjecture.

2.3.2 Effects of Folic Acid on folate metabolism and UMFA

At intakes above 200 µg, FA can appear in the circulation as UMFA. FA is absorbed by small intestinal epithelial cells (i.e., enterocytes) via the PCFT (3). At doses below 200 µg, FA the majority of FA metabolism occurs in the liver. At higher doses of FA (above 200 µg), free FA can passively diffuse through the enterocytes into the portal circulation, unchanged (3). At these higher concentrations, UMFA can circulate in the blood. Upon entering the cell, FA must be reduced to DHF via DHFR in order to be converted into an active folate substrate. Once it is converted to DHF it is free to be reduced again by DHFR to yield tetrahydrofolate (THF). Further SHMT mediated transformations yield 5,10-methyleneTHF. 5,10-methyleneTHF is reduced further by MTHFR to yield 5MTHF. DHFR in the liver has a relatively low capacity for FA reduction and hence, UMFA can be found in the circulation at high intake levels of FA (3).
Figure 2.4 FA and 5MTHF metabolism: Folic acid from supplements or fortified foods must be reduced by DHFR (Dihydrofolate reductase) to DHF (Dihydrofolate) before it can be used in the folate cycle. FA is a poor substrate for DHFR and has also been seen to downregulate DHFR expression. High levels of folate can potentially create a back log of DHF and other folate metabolites waiting to be reduced to THF (Tetrahydrofolate). It is argued that 5MTHF may be a better means of supplementation as it is already reduced and can be readily incorporated into the folate cycle. Reproduced with permission from the publisher De Gruyter (3) Copyright © 2013 De Gruyter

The role of UMFA in disease outcomes is controversial. Some studies have suggested an association of UMFA with the progression of (pre)neoplastic lesions, competition with folate for folate-dependent enzymes and transporter, and decreased natural killer cell cytotoxicity (2,3,15). However, it is unknown if these outcomes are specific to UMFA or due to high folate in general. After FA fortification in the US, vitamin users have significantly higher serum 5MTHF concentrations than before fortification (3). In Germany where there is no mandated FA fortification of food, it was found that supplemental FA intake correlated directly with increased 5MTHF blood concentrations (3). Obeid and Herrmann state that, “if FA were to cause harm by altering DNA synthesis or methylation, it is more likely that this would be caused by the predominant 5MTHF form or other forms with direct biological functions rather than by the minor amount of UMFA” (133). At present, the effects of high levels of reduced folate forms and
FA on the folate metabolic and one-carbon transfer reaction pathways have not been comprehensively examined.

2.3.2 Effects of Folic Acid on Folate Metabolism Proteins

2.3.2.1 Folic acid and transporters/carriers (PCFT, RFC, FR):

Many studies have attempted to elucidate the effects of FA supplementation on folate transporters \textit{in vivo}. Tactan et al. studied the effects of FA supplementation of 10 mg FA and 100 mg FA on the PCFT and RFC in the intestine of laying hens (111). They found that in the duodenum, uptake of FA decreased but mRNA levels of PCFT and RFC were not affected. In the jejunum, uptake and mRNA levels of PCFT and RFC were not affected. Dev et al. demonstrated that intestinal uptake of FA decreased in rats during acute FA supplementation (23). They observed a decrease in protein, but not mRNA, expression of PCFT and RFC. It is speculated that during FA supplementation, there might be post-transcriptional or translational adaptation of folate transporter genes. Jing et al. observed a non-significant decrease in mRNA expression of RFC in an avian model supplemented with 10 mg FA (compared to no supplementation). Interestingly, they found that hens supplemented with 5MTHF had significantly lower RFC mRNA expression than those supplemented with FA; however, the mRNA expression between hens supplemented with FA and 5MTHF did not differ (112, 113). In an \textit{in vitro} model, Ashokkumar et al. reported a decrease in the steady-state levels of protein and mRNA of hRFC as well as a decreased hRFC promoter activity with increased supplemental levels of FA in the media (114). In a folate deficient mouse model, Qiu et al. found a significant increase in FA transport which was characterized by increased mRNA levels of PCFT (115). Overall, it was found that FA supplementation decreased FA uptake or expression of PCFT, RFC, and FR proteins and mRNA (Table 2.7). Folate deficiency generally resulted in upregulation of FA
uptake or expression of PCFT, RFC, and FR proteins and mRNA. Beyond Jing et al.’s (112, 113) studies on RFC and PCFT mRNA expression in hens, no studies have characterized the effects of 5MTHF supplementation on expression and activity levels of folate transporters.

2.3.4 Folic acid and intracellular folate metabolism, methionine cycle, and DNA methylation

It has been found that extracellular folate levels may modulate SHMT expression and activities (116-118). One study found that high folate intake elevated cSHMT mRNA levels in endometrial and embryonic tissues (117). Another study found that folate deficient diets also elevated SHMT activity in chickens (118). This alludes to the possibility of other factors affecting the relationship between folate status and SHMT activity. In regards to MTHFR activity, it has been seen in a mouse model that high FA (20 mg FA/kg diet) consumption could result in reduced MTHFR protein and activity levels compared to control (2 mg FA/kg) (139).

In an in vitro model, Hayashi et al. investigated the effect of folate deficiency on the steady-state levels of genes regulating intracellular one-carbon metabolism using human HCT 116 and Caco-2 colon adenocarcinoma cell lines (110). In this in vitro model, folate depletion resulted in metabolic changes, which favoured the shuttling of folate toward the methionine cycle over the nucleotide synthesis pathway in HCT116 cells. However, in CaCo2 cells, folate depletion favoured nucleotide synthesis over biological methylation reactions. It is currently unknown what the consequences of 5MTHF supplementation are on the steady-state mRNA and protein levels of genes involved in folate metabolism and one-carbon transfer reactions.
### Table 2.7 Summary of supplementation studies on folate transporters

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Model</th>
<th>Treatment</th>
<th>Results</th>
<th>Overall</th>
</tr>
</thead>
</table>
| Ashokkumar et al. 2007 | Caco-2 HK-2             | 5 generations                      | **Treatments:** Oversupplemented: 100 µM  
Intermediate: 9 µM  
Low: 0.25 µM  
Treatments:  
1) Basal diet + 10.00 mg/kg FA  
2) Basal diet + 11.30 mg/kg 5MTHF | Oversupplementation decreased RFC, PCFT, and FR.  
Long-term oversupplementation of FA leads to downregulation of folate uptake and decreased mRNA levels of RFC, PCFT, and FR |
| Jing et al. 2009  | Shaver White hens      | 21 days                            | **Control:** Basal diet with no supplemental folate  
**Treatments:**  
1) Basal diet + 10.00 mg/kg FA  
2) Basal diet + 11.30 mg/kg 5MTHF | FA supplementation non-significantly lower RFC mRNA expression compared to control in jejunum  
5MTHF supplementation significantly lower RFC mRNA expression compared to control in the jejunum  
RFc mRNA expression not significantly different between FA and 5MTHF supplementation in the jejunum | FA or 5MTHF supplementation may lower RFC mRNA expression in the jejunum |
| Jing et al 2010    | Shaver White hens      | 21 days                            | **Control:** Basal diet with no supplemental folate  
**Treatments:**  
1) Basal diet + 10.00 mg/kg FA  
2) Basal diet + 11.30 mg/kg 5MTHF | 5MTHF supplementation significantly lowered PCFT mRNA expression in the jejunum compared to basal diet  
FA supplementation did not affect PCFT mRNA expression in jejunum compared to basal or 5MTHF diet | 5MTHF supplementation may downregulate PCFT mRNA expression in the jejunum |
| Dev et al. 2011    | Male albino rats (Wistar) | Acute: 10 days  
Chronic: 60 days  
**Control:** 2 mg/kg folic acid  
**Treatment:** 20 mg/kg folic acid | Acute treatment: Decreased PCFT and RFC | Not affected | Acute but not chronic, FA oversupplementation led to significant downregulation in intestinal folate uptake proteins but not mRNA |
| Tactan et al. 2012 | Shaver White hens      | 28 days                            | **Control:** Basal diet with no supplemental folate  
**Treatments:**  
1) Basal diet + 10 mg/kg FA  
2) Basal diet + 100 mg/kg FA | N/A | Duodenum: decreased FA uptake  
Jejunum: not affected |
<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Model</th>
<th>Treatment</th>
<th>Results of deficiency</th>
<th>Overall effect of deficiency compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Said et al., 2000</td>
<td>Sprague-Dawley Rat</td>
<td>6-8 weeks Control: 2 mg/kg folic acid Treatment: folate deficient diet</td>
<td>Increased RFC</td>
<td>Increased uptake and expression levels compared to control</td>
</tr>
<tr>
<td>Veedamali et al 2003</td>
<td>Caco-2 HuTu-80</td>
<td>5 generations post-confluency Control: 200 nM folic acid in folate deficient DMEM Treatment: Folate deficient DMEM</td>
<td>Increased RFC</td>
<td>Increased RFC expression</td>
</tr>
<tr>
<td>Qiu et al., 2007</td>
<td>Male C57BL/6 mouse</td>
<td>8 weeks Control: 2mg/kg folic acid Treatment: folate deficient</td>
<td>PCFT</td>
<td>Increased PCFT expression</td>
</tr>
<tr>
<td>Hayashi et al., 2007</td>
<td>Caco-2 HCT116</td>
<td>20 days Control: RPMI 1649 Treatment: Folate deficient RPMI</td>
<td>N/A</td>
<td>Increased intestinal PCFT expression</td>
</tr>
<tr>
<td>Liu et al., 2005</td>
<td>C57BL/6 mouse</td>
<td>8 weeks Control: 2mg/kg FA Treatment: folate free</td>
<td>Increased RFC</td>
<td>Increased intestinal RFC expression and increased FR-α coding mRNA expression</td>
</tr>
</tbody>
</table>
2.4 5MTHF: the case for its supplementation

FA has been seen to be more advantageous as a fortificant and supplement than reduced natural folates primarily due to its greater stability and lower cost, allowing for a longer shelf-life. Until recently, 5MTHF, a reduced folate, was available as mixed diastereoisomers (L and D forms). Mixed diastereoisomers allowed for a longer shelf-life but possessed half the biological activity compared to FA (119). Now, 5MTHF is available commercially as a calcium salt in the L (active) form (24) and has been shown to be as stable as the diastereoisomers (120). Hereafter, unless otherwise stated, 5MTHF will refer to L-5MTHF.

2.4.1 5-MTHF-Ca (C₂₀H₂₃CaN₇O₆; Molecular weight: 497.5)

L-5MTHF, [6S]-5-methyltetrahydrofolate, Metafolin®, Levometafolin,

FA and 5MTHF can both be found commercially over-the-counter in doses of 200 µg, 400 µg, 800 µg, and 1,000 µg. As a supplement, FA is widely distributed and is easily accessible over-the-counter at most drug stores. 5MTHF is also found commercially in a crystalline calcium salt form (Metafolin®) and likely to be found in organic or natural health product stores, or online. Metafolin® is patented by Merck Inc. and is distributed to supplemental (eg. Genestra Brands, ProThera Inc. Pure encapsulations, and Thorne) and pharmaceutical companies (eg. PamLab and Bayer Schering Pharma). These supplemental brands are available for purchase in Canada as well as the US and widely available online.

Pharmaceutically, 5MTHF is found in birth control pills, Beyaz and Safyral, manufactured by Bayer Schering Pharma and contains 451 µg of 5MTHF (121, 122). 5MTHF is the main component of Deplin®, a pharmaceutical product marketed by PamLab as a new medical food therapy to provide adjunctive support for patients with major depressive disorder or schizophrenia (122). Doses are available at 7.5 mg or 15 mg of Metafolin® (122). PamLab also
markets Neévo® or NeévoDHA® as a prenatal medical food. Prenatal vitamins contain 0.8 to 1.0 mg of FA while Neévo® contains 1.13 mg of 5MTHF and 0.4 mg FA. Also, prenatal vitamins contain 0-12 µg of B12 while Neévo® and NeévoDHA® contain 500 µg and 1000 µg of B12 respectively. Other medical foods on the market include Metanx®, Cerefolin®, or CerefolinNAC® (Pamlab).

While Metafolin® (L-5-MTHF-Ca) is advertised as a natural folate, it is derived from the synthetic FA. It is obtained through a series of reduction and condensation reactions starting with FA (123) (refer to figure 2.5). L-5-MTHF-Ca is stable in storage for 48 months when processed for supplemental tablets. When it is in a microencapsulated form with ascorbate as an antioxidant, it can be used as a fortificant (17, 123).

In the US, 5MTHF-Ca has been determined as GRAS (Generally Recognized as Safe) for its intended use (17, 123) and can be used as a dietary ingredient (17, 23). The EFSA (European Food Safety Authority) has also supported the use 5MTHF-Ca in foods for specific nutritional uses with an upper limit of 1 mg/day for adults (124). Again, the upper limit of 1000 µg was applied due to the paucity of data on the relationship between folate and progressive neuropathy caused by B12 (124).

### 2.4.2 Arguments for 5MTHF supplementation

5MTHF supplementation may provide benefits for those with MTHFR polymorphisms (explained below in section 2.4.3) or those who are B12 deficient. However, other broad arguments for 5MTHF as a superior supplemental form to FA are: 1) 5MTHF is immediately available for use by the cells (whereas FA must be reduced twice to become THF) and thus making it more bioavailable (2); 2) supplementation of 5MTHF will result in much less UMFA in the blood; 3) 5MTHF will prevent masking B12 deficiency; and 4) 5MTHF may be associated
with a reduced interaction with drugs that inhibit DHFR (compared to FA) (24). Studies have shown the bioavailability of FA and 5MTHF to be approximately equivalent with comparable physiological activity (24). Furthermore, long-term supplementation in human studies showed that 5MTHF and FA similarly decreased homocysteine concentrations and similarly increased RBC and plasma folate concentrations (26).

**Figure 2.5 Flow chart of L-5MTHF-Ca production** Folic acid is reduced to (6R,S)-tetrahydrofolate (THF). The diastereoisomers are selectively crystalized for the (6S) form. (6S)-THF is then methylated and subsequently bonded to a Ca$^{2+}$ to yield the L-5MTHF-Ca salt. Reproduced with permission from (124) FAO © 2006

2.4.3 5MTHF and MTHFR SNP

Studies have shown polymorphisms of several genes involved in folate metabolism to greatly affect health (2, 61). In this regard, the MTHFR C677T polymorphism has been
extensively studied. The frequency of 677TT genotype varies across different ethnic groups and geographical locations; approximately 10% of the Caucasian and Asian population are homozygous carriers while the Hispanic population has a higher frequency (24). The prevalent concern for those with \textit{MTHFR} C677T polymorphism is the reduced ability to recycle folate within the cell. Studies have shown the potential for increases in total homocysteine (tHcy) levels, and DNA hypomethylation in those with the \textit{MTHFR} C677T polymorphism (39). Many believe that 5MTHF supplementation allows the folate cycle to bypass the need for MTHFR, allowing for consistent SAM regeneration and DNA methylation (refer to figure 2.4). However, the effects of the \textit{MTHFR} C677T polymorphism on folate biomarkers are not always consistently observed (125,126).

\textbf{2.4.4 5MTHF and B12}

The haematological and neurological connection between B12 and FA have been discussed earlier. In terms of B12 and 5MTHF, there is not yet convincing evidence for 5MTHF as an advantageous supplemental/fortificant form over FA. Many propose that supplementing with 5MTHF will create a ‘methyl trap’ during B12 deficiency. The ‘methyl trap’ refers to a halt in the folate pathway at MS during B12 deficiency. MS requires B12 to function. Without MS activity, 5MTHF will not be able to participate in the transmethylation of homocysteine to methionine in the generation of SAM nor will it be able to be involved in nucleotide biosynthesis (refer to figure 2.2). This would result in megaloblastic anemia, a clinical marker of B12 deficiency.

While arguments for 5MTHF as a superior supplemental form are logical, the urgency to switch from FA to 5MTHF is not grounded in convincing research. While many argue that megaloblastic anemia is an important clinical marker for B12 deficiency and that high intakes of
FA increase the risk of masking B12 deficiency, a recent study in the US by Qi et al (129) did not find an increased risk of masking B12 deficiency post FA fortification. This suggests that low B12 can still be clinically seen through megaloblastic anaemia or macrocytosis despite higher intakes of FA. Furthermore, a study found that in the elderly with low B12 status, high folate status is associated with higher risk of cognitive impairment than in those with low B12 status and ‘normal’ folate status (41). If this is true and if 5MTHF is at least as effective as FA in increasing folate levels, will supplementing with 5MTHF rather than FA be more protective against cognitive impairment? FA supplementation did not increase the prevalence of adverse effects seen with B12 deficiency and thus 5MTHF supplementation may not address this issue. Perhaps the key issue for health providers and public health policy makers is the need to address low B12 status.

2.4.5 Effects of 5MTHF on folate metabolism

As previously discussed, 5MTHF is the active and predominantly circulating form of folate in the blood and tissue stores. Other than Jing et al. (112, 113), no studies have investigated the effect of 5MTHF supplementation on the expression of mRNA and proteins of genes involved in folate metabolism. Jing et al., (112 113) found that compared to a basal diet, 5MTHF supplementation resulted in lower mRNA expression of PCFT and RFC. When compared to equimolar FA supplementation, there was no difference in PCFT and RFC mRNA expression.

2.5 Folic Acid vs. 5MTHF

Several human studies have directly compared FA and 5MTHF (Table 2.9). The majority of these studies were clinical studies that focused on biomarkers of folate status and tHcy status. Overall, these studies found 5MTHF supplementation may be at least as effective as FA
supplementation in raising folate status or lowering tHcy status compared to FA. Study results differed depending on the sample population. Comparable effects of FA vs. 5MTHF on folate and tHcy status were generally seen in those from the general healthy population, however measures in pregnant or lactating women differed when comparing FA and 5MTHF.

Houghton’s (125) study in lactating women in a fortified country found that at week 16, those on 5MTHF supplements had significantly higher RBC folate concentrations after correcting for baseline compared to those supplemented with FA or on placebo. However, Lamers et al (130), using the same supplemental doses, found that there was no difference in RBC folate concentrations at the end of 24 weeks between FA and 5MTHF. Pentieva et al. (126) found that the bioavailability for FA and 5MTHF were the same for men and women. De Meer et al. (131) found lower FA absorption (but not 5MTHF) in those ≥50 years compared to those between 18-30 years. Further, they found that the young aged group compared to the older aged group had higher absorption and elimination of FA compared to 5MTHF after 5 weeks of supplementation.

No studies have assessed the bioavailability of these supplements in pregnant women. Finally, Houghton (125) and Pentieva (126) found no differences between FA and 5MTHF supplementation on UMFA for participants with the homozygous \textit{MTHFR} C677T gene.

However, Pentieva administered supplements of equivalent mass rather than molar, which may affect the results.
### Table 2.9 Studies comparing FA and 5MTHF supplementation in humans

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Study population</th>
<th>Treatment</th>
<th>Measures</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fohr et al. 2002</td>
<td>Non-pregnant healthy women (19-39 years) n=160</td>
<td>4 week washout 8 week treatment</td>
<td>Plasma tHcy concentrations (lowering potential), plasma folate, and folate metabolites in plasma</td>
<td>At baseline, TT (homozygous) genotypes had significantly lower plasma folate than CT (heterozygous) and CC (wild-type) and At baseline, there were no significant differences in plasma tHcy and RBC folate concentrations between genotypes By week 8, those with with CT and CC genotypes and supplemented FA had reduced plasma tHcy concentrations compared to baseline By week 4, those with CT genotypes and supplemented 5MTHF had significantly reduced plasma tHcy concentrations compared to baseline By week 9, those with TT genotype and no folate had significantly increased plasma tHcy concentrations compared to baseline Increased RBC folate concentration significantly different than placebo for both groups.</td>
</tr>
<tr>
<td>Litynski et al. (2002)</td>
<td>Males (n=32) and females (n=8) (19-69 years)</td>
<td>7 weeks of treatment 24 weeks post treatment</td>
<td>Plasma homocysteine concentrations</td>
<td>For all genotypes, both FA and 5MTHF reduced tHcy concentrations by week 7 At week 24 post treatment, only 5MTHF supplementation in the homozygous group had sustained lower plasma tHcy concentrations. All other genotypes and folate types were not significantly different from baseline For all genotypes and treatments, plasma folate was higher at week 3, 7 and 24.</td>
</tr>
<tr>
<td>Venn et al. 2002</td>
<td>Women of childbearing age (18-49 years) n=104</td>
<td>24 week</td>
<td>Plasma folate and RBC folate concentrations</td>
<td>Plasma folate and folic acid groups increased for both 5MTHF and FA.</td>
</tr>
<tr>
<td>Venn et al. 2003</td>
<td>Healthy men and women (&gt; 18 years) n=155</td>
<td>24 week</td>
<td>Plasma tHcy, Plasma folate, and RBC folate concentrations</td>
<td>FA and 5MTHF groups both had significantly lower plasma tHcy The mean plasma tHcy reduction was significantly greater in the 5MTHF group than the FA group No significant difference in plasma folate or RBC folate concentrations between FA and 5MTHF</td>
</tr>
<tr>
<td>Lamers et al. 2004</td>
<td>Female participants n=114</td>
<td>24 week</td>
<td>Plasma tHcy, plasma folate concentrations</td>
<td>No significant difference in decrease in tHcy between 3 supplemental groups Increase in plasma folate for 400 µg and 416 µg 5MTHF significantly higher than 208 µg group</td>
</tr>
<tr>
<td>Study</td>
<td>Population</td>
<td>Treatment</td>
<td>Study Design</td>
<td>Outcome</td>
</tr>
<tr>
<td>-------</td>
<td>------------</td>
<td>-----------</td>
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</tr>
<tr>
<td>Pentieva et al 2004</td>
<td>Males (18-25 years) n=21</td>
<td>Placebo + 400 µg folic acid Placebo + 416 µg 5MTHF Placebo + 208 µg 5MTHF</td>
<td>10 hr. cross over with 1 week washout period</td>
<td>Plasma folate concentrations</td>
</tr>
<tr>
<td>De Meer et al 2005</td>
<td>Young adults (18-30 years) and middle aged adults (≥50) n=24</td>
<td>Placebo: capsule Treatment: 500 µg FA capsule 500 µg 5MTHF capsule</td>
<td>5 weeks Placebo: gelatin capsule Treatment: 400 µg (908 nm) FA 454 µg (908 nm) 5MTHF</td>
<td>% changes in apparent absorption, true absorption, and rate constant of elimination. % changes in plasma Fasting tHcy, tMTHF, and total folate concentrations</td>
</tr>
<tr>
<td>Houghton et al. 2006</td>
<td>Lactating women after delivery n=72</td>
<td>Placebo: multivitamin and mineral supplement + 0 µg folate Treatment: Placebo supplement + Folic acid (400 µg/d, 906 nmol/d) Placebo supplement + 5MTHF (416 µg/d, 906 nmol/d)</td>
<td>16 week RBC and plasma folate concentrations and plasma tHcy concentrations Folate forms</td>
<td>At week 16, 5MTHF treatment had higher RBC folate concentrations than folic acid and placebo after correcting for baseline. Both treatments and placebo had RBC folate concentrations lower at week 16 compared to week 4 after correcting for baseline. Plasma folate concentrations for folic acid and 5MTHF higher than placebo group at week 16. No differences in plasma tHcy between groups at week 16. Higher 5-FormylTHF in RBC for FA group compared to placebo and 5MTHF groups. No difference in UMFA between groups. MTHFR 677C&gt;T polymorphism did not influence blood folate</td>
</tr>
<tr>
<td>Green et al 2013</td>
<td>Healthy men and women (18-45 years old) n=45</td>
<td>Placebo: wheat rolls + 0 µg folate Treatment: Wheat rolls with 400 µg FA Wheat rolls with 452 µmicroencapsulated 5MTHF</td>
<td>16 weeks consuming 1 wheat roll per day Erythrocyte folate and plasma folate concentrations at week 8 and week 16</td>
<td>By week 16, FA and 5MTHF fortified wheat rolls had significantly higher folate concentrations in erythrocyte and plasma. No significant difference between FA and 5MTHF fortified rolls at week 16. No significant difference between all groups at week 8 and baseline.</td>
</tr>
</tbody>
</table>
Few animal and *in vitro* studies have compared the effects of FA vs. 5MTHF supplementation on folate absorption (Table 2.10 and table 2.11). An *in vivo* model by Jing et al (112, 113; table 2.8) investigated the effect of FA and 5MTHF on folate absorption in the jejunum of laying hens. They found that 5MTHF supplementation significantly lowered RFC and PCFT mRNA expression compared to the control diet. FA supplementation did not result in any differences between in mRNA expression compared to the controlled diet. However, no difference was observed between 5MTHF and FA supplementation.

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>Model</th>
<th>Treatment</th>
<th>Results</th>
</tr>
</thead>
</table>
| N/A | N/A | N/A | FA supplementation non-significantly lower RFC mRNA expression compared to control in jejunum  
5MTHF supplementation significantly lower RFC mRNA expression compared to control in jejunum  
RFC mRNA expression not significantly different between FA and 5MTHF supplementation in the jejunum | FA and 5MTHF supplementation may lower RFC mRNA expression in the jejunum |
| Jing et al. 2009 | Shaver White hens | Basal diet with no supplemental folate  
Treatments:  
1) Basal diet + 10.00 mg/kg FA  
2) Basal diet + 11.30 mg/kg 5MTHF | N/A | FA supplementation significantly lowered PCFT mRNA expression in the jejunum compared to basal diet  
FA supplementation did not affect PCFT mRNA expression in jejunum compared to basal or 5MTHF diet |

Wang and Fenech (73; table 2.11) compared effects of FA and 5MTHF supplementation on DNA damage and cell death in human lymphocytes. 5MTHF was not more efficient than FA in
preventing human lymphocyte genomic instability in vitro. 120 nM of FA was significantly more effective than 12 nM 5MTHF in minimizing micronucleus formation (a measure of DNA damage). 120 nM FA also had higher total viable cell counts than 120 nM 5MTHF. They proposed a few plausible explanations for these observations. One explanation was that lymphocytes in culture took up FA more efficiently than 5MTHF. Another explanation is that 5MTHF was unable to participate in the thymidylate cycle due to the high levels of choline and methionine (73, 132). Choline concentrations in the culture media was much higher than what is found physiologically (21.5 µM vs. 6-7 µM). Choline can be oxidized to betaine, which may indirectly inhibit the conversion of 5MTHF to THF and 5, 10-methylenetetrahydrofolte. This could indirectly block the involvement of 5MTHF in thymidine synthesis, which could lead to DNA damage (73, 131). Further, the supraphysiological concentration of methionine (100 µM) in the culture medium might have reduced the need for 5MTHF to regenerate methionine from homocysteine. Thus, in this in vitro setting, 5MTHF does not participate in the thymidylate cycle (73, 132).

Table 2.11 Studies comparing FA and 5MTHF in vitro

<table>
<thead>
<tr>
<th>Study (references)</th>
<th>(Model)</th>
<th>Treatment</th>
<th>Measures</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang and Fenech 2003</td>
<td>Human lymphocytes cultured from primary cells (n=8 women aged 40-48 years)</td>
<td>3, 6, and 9 days Treatments: 1) 12 nM FA 2) 12 nM 5MTHF 3) 120 nM FA 4) 120 nM 5MTHF</td>
<td>DNA damage: - Micronucleated BN cells - Nuclear buds - Nucleoplasmic bridges - Micronucleated Mono cells Cell Viability - Nuclear division index - Viable cell count (days 3, 6, and 9) - Necrosis - Apoptosis</td>
<td>- 120 nM FA significantly lower micronucleated BN cells compared to 120 nM 5MTHF - 120 nM FA significantly higher viable cell count than 12nM 5MTHF - 120 nM FA significantly lower apoptosis than 12 nM 5MTHF</td>
</tr>
</tbody>
</table>
There are no studies that specifically interrogate the effects of 5MTHF supplementation in those with the *MTHFR C677T* polymorphism. Also, studies comparing the effect of FA vs.
5MTHF supplementation on raising blood folate concentrations in those with the *MTHFR C677T* polymorphism are few and the results are equivocal. Houghton (125) and Pentieva (126) found no differences between FA and 5MTHF supplementation on RBC folate status in participants with the *MTHFR C677T* polymorphism. However, Pentieva (126) administered supplements of equivalent mass rather than moles. This means that less 5MTHF moles were provided compared to FA and could result in different outcomes than when equimolar amounts are administered.

Two studies have shown *MTHFR* genotype interaction with folate form. Fohr (127) found that form of folate supplementation may have differential effects on reducing plasma tHcy concentrations. FA supplementation reduced plasma tHcy concentrations to a greater extent than supplementation with 5MTHF for all genotype groups. Although not significant, they also found that subjects with the *TT* genotype had the greatest reduction of tHcy concentrations in the plasma. This study administered equimolar doses of FA and 5MTHF, but 5MTHF was administered as a racemic mixture (50% 6S and 50% 6R) rather than only the 6S active isomer. Litynski et al (128) similarly investigated the effect of FA and 5MTHF on the *MTHFR C677T* homozygous vs. wildtype using the racemic mixture of 5MTHF, but administered doses with equivalent mass rather than moles. They found that while both 5MTHF and FA reduced tHcy levels significantly for both genotypes with no difference between treatments, only the homozygous (*TT*) group supplemented with 5MTHF had sustained lower tHcy levels at 24 weeks post treatment.
2.6 Summary and Research Gaps

Folate plays an important role in human health and disease. FA fortification and periconceptional FA supplementation have significantly decreased NTD rates. However, owing to widespread FA supplement use and FA fortification, the intakes and blood levels of folate and FA have significantly increased (2, 3, 4). Several lines of evidence suggest potential detrimental effects of high folate status (15, 39) and the dramatic increased FA intake and UMFA in the circulation have been purported to be responsible for these adverse health effects. The use of 5MTHF supplementation instead of FA has been promoted as a means of avoiding the appearance of UMFA in the circulation, thereby reducing the risk of adverse health outcomes. Although comparable effects of FA and 5MTHF supplementation on increasing blood folate concentrations and lowering tHcy levels have been reported, no head-to-head comparisons have been done with regard to potential differential effects between FA and 5MTHF supplementation on intracellular folate metabolism and one-carbon transfer reactions.

While studies have attempted to tease apart the complexities of folate deficiency and FA supplementation on folate metabolism and clinical outcomes, the optimal dose and timing of FA supplementation has yet to be determined. To date, the effect of 5MTHF supplementation on folate metabolism and one-carbon transfer reactions is largely unknown. A number of clinical studies have assessed the bioequivalence of FA compared to 5MTHF on the change in blood folate levels. They have found a slight advantage of 5MTHF supplementation or an equivalent impact of 5MTHF and FA supplementation on raising RBC folate concentrations (125, 119, 128). Only 3 studies have compared the effects of equimolar FA and 5MTHF supplementation on folate absorption. In a hen model, 5MTHF and FA supplementation had similar effects on folate transporter RFC and FR mRNA expression (122). However, 5MTHF supplementation resulted in
greater PCFT down regulation compared to FA supplementation (123). No studies to date have assessed the differential impact of FA and 5MTHF supplementation on genes and proteins involved in intracellular folate metabolism and one-carbon transfer reactions including DNA synthesis and methylation and on health outcomes such as cancer. My thesis aims to address some of these research gaps.
Chapter 3  Rationale, Objectives, Hypothesis, and Significance

3.1  Rationale

FA fortification and supplementation have significantly increased the intake and blood levels of folate and FA in North America (4). Although FA fortification has achieved its primary objective in reducing NTD rates and although FA supplementation may provide other health benefits, an emerging body of evidence suggests that high folate status (mainly from FA supplementation) may be associated with unwanted adverse health outcomes (15). The tolerable upper limit (UL) for FA was set by the Institute of Medicine at 1 mg/day to reduce the risk of masking B12 deficiency (1). It is extremely difficult to achieve high intakes of folate from natural sources alone and hence, no UL for natural folates have been established yet. As 5MTHF is naturally found in foods and in circulation in the body it has been promoted as a safer form of folate supplementation compared with FA, even though there is no convincing evidence to support this health claim. After the emergence of L-5MTHF-Ca on the market at similar doses of FA, most countries and governing bodies have yet to establish a UL for the supplemental form of 5MTHF. Currently, only the European Food Safety Authority (EFSA) has established an upper limit of 1 mg/day for adults for 5MTHF (124). This limit was set even though there is little evidence showing its efficacy or safety over FA.

Before recommending 5MTHF as an alternative form of providing supplemental levels of folate, the effects of 5MTHF supplementation in comparison to FA supplementation on folate metabolism and 1-carbon transfer reactions must be more understood. Adverse effects of high folate status may not be limited to high FA intake and elevated levels of plasma UMFA. These adverse effects may also arise from high intakes of 5MTHF (3). Human studies comparing FA to 5MTHF supplementation only investigated their comparative effects on elevating RBC and
plasma folate and lowering homocysteine concentrations (refer to table 2.9). Only 3 studies to date have interrogated the effects of equimolar FA and 5MTHF supplementation on biochemical outcomes (73, 111, 112; refer to tables 2.10 and 2.11). More work needs to be done in order to assess the differential consequences involved with nucleotide biosynthesis and biological methylation reactions at the cellular level.

3.2 Objectives

To determine if FA supplementation confers different effects on cellular processes and folate pathways from 5MTHF supplementation on cellular growth and proliferation and gene expression of folate transporters and enzymes involved in folate metabolism and one-carbon transfer reactions in an in vitro model using human colon cancer cell lines.

Specific Aims:

In an in vitro model using human Caco-2 and HCT116 colon cancer cell lines:

   a. To compare the effect of FA vs. 5MTHF supplementation on cellular growth and proliferation;

   b. To compare the effects of FA vs. 5MTHF supplementation on gene expression of folate transporters and enzymes involved in folate metabolism and one-carbon transfer reactions;

   c. To compare the effect of FA vs. 5MTHF supplementation on global DNA methylation; and

   d. To determine if there is a threshold concentration where no differences in the aforementioned outcomes exist between FA and 5MTHF supplementation;
3.3 **Hypothesis**

Higher concentrations of 5MTHF (500 nM and 1000 nM) will have similar effects as equimolar levels of FA on cellular growth rate and proliferation, folate metabolism, and one-carbon transfer reactions.

3.4 **Expected outcomes**

1) With increasing supplementation for both FA and 5MTHF, Caco-2 and HCT116 cell lines will grow faster.

2) Due to similar binding affinities of PCFT for FA and 5MTHF intracellular folate concentrations will be different at low doses. However, higher doses of supplementation may lead to differences in intracellular folate concentrations because FA is a poor substrate for DHFR and FPGS. This may lead to increased efflux due to limited reduction of FA to DHF and therefore limited substrates for FPGS to add polyglutamate chains for folate retention.

3) Gene expression:
   a) **Folate Transporters:** Regardless of FA or 5MTHF, supplementation of FA and 5MTHF will significantly decrease steady-state mRNA expression levels of genes involved in folate absorption and uptake compared with the control;
   b) **Intracellular folate metabolism and one-carbon transfer reactions:** Regardless of FA or 5-MTHF, supplementation of FA and 5-MTHF will both significantly influence steady-state mRNA expression levels of genes involved in intracellular folate metabolism and one-carbon transfer reactions;
   c) **Global DNA methylation:** Regardless of FA or 5MTHF, supplementation of FA and 5MTHF will increase global DNA methylation
d) Steady-state mRNA expression levels of the selected genes involved in folate absorption, uptake and intracellular folate metabolism and one-carbon transfer reactions will not be significantly different between FA and 5MTHF.

3.5 **Significance**

Clinical studies have shown the efficacy of 5MTHF in improving folate status, thereby suggesting that it might be at least as efficient as FA supplementation in treating folate deficiency and reducing the risk of certain folate deficiency-associated disorders such as NTDs. Many nutritionists are urging the use of 5MTHF over FA as it is a natural form of folate found in foods and to avoid negative outcomes associated with high intakes of FA and high plasma levels of UMFA. These recommendations are based on limited scientific evidence and it is still uncertain whether supplementation with 5MTHF poses fewer negative health consequences than that with FA. Before 5MTHF is used widely to replace FA in supplementation and fortification, it is important to understand how both forms compare at the cellular level. This project will determine how FA supplementation compares to equimolar 5MTHF supplementation in regards to folate metabolic pathways, one-carbon reaction pathways, and global DNA methylation. Considering the high intakes and blood levels of folate and FA in the Canadian population as a result of FA fortification and widespread supplemental use, the proposed research will provide a framework for future research on the efficacy and safety of folate supplementation. This will help determine the most effective and safest recommendations for folate supplementation.
Chapter 4  Comparative studies on the supplemental effects of FA vs. 5MTHF in human colon cancer cells

Folate is undoubtedly integral for the healthy function of cells as it is involved in DNA synthesis, one carbon metabolism, and DNA methylation. Since the mandated FA fortification in North America, blood folate concentrations have increased and the number of pregnancies affected by an NTD outcomes has decreased (4). The IOM recommends adults to consume 400 DFEs of folate daily with an UL of 1,000 µg for FA only. The UL was set based on the potential for FA to mask B12 deficiency, which could result in long term negative cognitive and neuropsychiatric outcomes. However, emerging evidence suggests that masking B12 deficiency may not be the only concern of high FA intakes. Many lines of evidence, mostly from animal models, suggest that high FA intakes may result in expedited cancer onset, perturbed epigenetic mechanisms, and negative health outcomes in offspring (2, 3, 15, 36, 39). Many are now advocating for the use of 5MTHF in supplements and fortificants because it possesses coenzyme activity once it is absorbed and because it is the predominant circulating form in the blood. However, it is unknown if 5MTHF is truly superior to FA as a supplement nor is it known if molar equivalent doses of 5MTHF confer any risks. The aim of these studies is to provide proof that studies interrogating 5MTHF must be conducted before it is left unregulated in the market.

It has previously been shown that folic acid supplementation and high intracellular folate concentrations are associated with modulated mRNA and protein expression levels within cell culture. Many of these studies examined the effect of folate deficiency (0 nM FA and folate in cell culture medium) compared to regular cell culture medium. Regular RPMI 1640 cell culture medium contains 2.3 µM of FA and is of higher concentrations than found physiologically in humans. Other cell culture mediums, such as DMEM, have double the
concentration of FA. In order to mimic more physiological conditions, Caco-2 and HCT116 cell lines were acclimatized to growing in 50 nM FA cell culture medium before starting supplemental treatments of 5 nM, 50 nM, 500 nM and 1000 nM concentrations of FA or 5MTHF.

4.1 Specific Objective and Hypotheses

Objectives

- To determine if FA supplementation confers different cellular and metabolic effects from 5MTHF supplementation on cellular growth and proliferation and gene expression of folate transporters and enzymes involved in folate metabolism and one-carbon transfer reactions in an in vitro model.

Hypotheses

- With increasing supplementation of the same folate form, intracellular folate concentrations and cell growth will differ at higher levels of supplementation.
- Between FA vs. 5MTHF, intracellular concentrations and cell growth may differ at high levels of supplementation due to DHFR being saturated by FA.
- The steady-state mRNA expression levels of genes encoding PCFT, RFC, and FR will decrease with increasing supplemental levels regardless of folate form.
- The steady-state mRNA expression levels genes encoding enzymes involved in intracellular folate metabolism and one-carbon transfer reactions and extent of global DNA methylation will not differ between FA and 5MTHF.

4.2 Materials and Methods

4.2.1 Cell lines and Cell Culture

Human HCT116 and Caco-2 colon adenocarcinoma cells were selected for the study (Table 3.1). These cell lines, Caco-2 in particular, were chosen as they have been extensively used to study human intestinal transport (110, 137). Further, other than the liver, FA absorption and metabolism occurs in the intestinal mucosal cells thus the effects of folate supplementation observed in these studies can be related further for future in vivo studies. HCT116 and Caco-2
cells, purchased from the American Type Culture Collection (Rockville, MD), were cultured in standard RPMI 1640 containing 2.3 µM FA (Wisent Bioproducts, Quebec, Canada) until confluency. Cells were then split and cultured in folate free RPMI 1640 (Wisent Bioproducts, Quebec, Canada) supplemented with 50 nM FA (herein 50 nM FA; control FA concentration) for 5 days before starting supplemental treatments of varying concentrations of FA or 5MTHF. At day 5 of acclimatization, medium was removed and replaced with folate free RPMI 1640 supplemented with either 5 nM FA (herein 5 nM FA), 50 nM FA, 500 nM FA, 1000 nM FA, or folate free RPMI 1640 supplemented with equimolar concentrations of 5MTHF (Metafolin®, Merck Eprova) from a 200 nM 5MTHF stock. These cells were harvested at day 0, day 7 and day 14 for intracellular folate assay and qRT-PCR. Growth medium was supplemented with 10% fetal bovine serum, FBS, 1% penicillin-streptomycin and 0.1% fungizone (all from Wisent Bioproducts, Quebec, Canada). Cells were maintained in standard conditions with incubation at 37º, 95% humidity, and 5% CO₂. Cell medium was changed every 3-4 days and passaged when 80% confluent.

Regular RPMI 1640 cell culture medium contains 2.3 µM of FA, which is much higher than normal physiological concentrations found in human plasma. Other cell culture mediums, such as DMEM, have even higher concentration of FA. In order to mimic more physiologically and nutritionally relevant concentrations, we used the approximate median human population serum folate concentration (50 nM) (106) as the control FA concentration in culture media. We then decided to supplement with 10X and 20X (500 nM and 1000 nM) the control concentration to determine the effects of supraphysiological intakes and plasma concentrations. Previous animal studies have supplemented diets with 10X the FA requirement to observe an effect and to mimic extreme supplemental levels found in the population (129, 144-145). As conditions in an
in vitro setting induce amplified cellular growth and needs, we chose 20X to accommodate for that. In addition, we used a deficient condition which is 0.1X the control concentrations (instead of 0 times) to witness low levels of folate supplementation and minimize growth retardation and death.

Table 4.1 Phenotypic and molecular characteristics of Caco-2 and HCT 116 (134, 135)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Caco-2</th>
<th>HCT 116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Colorectal adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td>Epithelial-like</td>
<td></td>
</tr>
<tr>
<td>Growth Properties</td>
<td>Adherent</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Male adults</td>
<td></td>
</tr>
<tr>
<td>Organism</td>
<td>Homo sapiens (Human)</td>
<td></td>
</tr>
<tr>
<td>Molecular features</td>
<td>TGH81 and TGFβ2 positive</td>
<td>Positive: Retinoic acid binding protein 1 and 2</td>
</tr>
<tr>
<td>Gene Mutations</td>
<td>MLH1, MSH3 A8, MSH6 C8, K-RAS, p14ARF, p16, BAX G8, β-catenin, DCC, Axin2, CBF2, E2F4, GRK4, Helicase q1, MBD4 10A, RAD50 9A, RIZ 9A, TGFBR-II</td>
<td>P53</td>
</tr>
</tbody>
</table>

200 µM 5MTHF stock

9.95 mg of 5MTHF Ca+ salt (Metafolin®, Merck Eprova MW 497.5 g/mol) was dissolved in 10 mL 1% Ascorbate in DPBS (Wisent Biosolutions, Quebec, Canada) to make a 2mM stock. This solution was filtered and sterilized using a syringe filter system. The stock was aliquoted to 500 µL and kept at -20 °C. At time of use, 1 aliquot of 500 µL was diluted into DPBS 1:10 to make 200 µM stocks aliquoted into 1 mL volumes and kept at -20 °C. To reduce
oxidation in the media and protect from light, 5MTHF was thawed and freshly diluted into folate free RPMI 1640 at each time of use.

4.2.2 Growth Curves

At the end of the 5 day of acclimatization in the 50 nM FA medium, 1.5 x 10⁶ (Caco-2) or 2 x 10⁶ cells (HCT116) were counted and seeded in 100 mm diameter plates containing either 5, 50, 500, or 1000 nM of FA or 5MTHF in triplicate. Cells were split and counted using Vi-CELL Cell Counter and Cell Viability analyzer (Beckman Coulter Inc. Brea, California, USA) every 3 to 4 days for 16 days.

4.2.3 Intracellular Folate Assay

Intracellular folate concentrations were determined by microbiological standard microtiter plate assay using Lactobacillus rhamnosus (formally known as Lactobacillus casei) (ATCC# 11578, Manassas, VA, USA) as described previously (136). In brief, 10 x 10² cells were harvested, washed three times with PBS and resuspended in 1 mL 1% ascorbic acid with PBS. Cells were sonicated on ice for 15 seconds 3 times. Cell lysates were diluted with equal volumes of 0.1M K₂HPO₄ and incubated with chicken pancreas conjugase at 37ºC for 2 hours. Aliquot of cell lysate in duplicate were incubated with Lactobacillus rhamnosus in mylar-sealed 96-welled microplates overnight for 16-18 hours. The turbidity of the media was measured spectrophotometrically at wavelength at 595 nm a standard curve was generated against a standard of known FA concentrations. The assay was repeated 3 times for each treatment.

4.2.4 mRNA expression via qRT-PCR

4.2.4.1 Total RNA extraction

Cells were harvested from confluent plates of cells from each treatment group at each time point. Total RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Mississauga, Canada)
according to the manufacturer’s protocol. Briefly, cells were washed 3 times with 10 mL of DPBS. Cells were then lysed and scraped directly on confluent plates using 1 mL of QIAzol Lysis Reagent (Qiagen Inc., Mississauga, Canada) and a cell scraper. Cells were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complex. 200 μL of chloroform was added and the tube was shaken vigorously for 15 seconds. The homogenate was then incubated for 3 minutes at room temperature and then centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase of the cells was transferred to a new tube and an equal volume of 70% ethanol prepared with RNase free water was added. The sample was mixed thoroughly and 700 μL of the sample was transferred to an RNeasy mini spin column placed into a 2 mL collection tube. The sample was centrifuged for 15 seconds at 10,000 RPM at room temperature and the flow-through discarded. 350 μL of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 RPM to wash the membrane and flow-through discarded. 10 μL of DNase 1 stock solution was added to 70 μL of RDD buffer and was mixed gently by inversion. 80 μL of the DNase incubation mix was added directly to the RNeasy spin column and was placed on the benchtop for 15 minutes. 350 μL of RW1 buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10 000 RPM and the flow-through was discarded. 500 μL of RPL buffer was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 RPM to wash the membrane and the flow-through was then discarded. The RNeasy spin column was placed in a new 1.5 mL collection tube and 30 μL of RNase-free water was added directly to the spin column membrane, the RNA was eluted via centrifugation for 1 min at 10,000 RPM.
4.2.4.2 cDNA production via Reverse Transcription

cDNA generation was performed all on ice. Stock RNA concentration and RIN (RNA Integrity Number) was measured using nanodrop and 1200 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA was diluted to 150 ng/µL concentrations for template RNA. Reverse transcription to cDNA was done using the Quantitec Reverse Transcription Kit (Qiagen). 2 µL of 7X gDNA wipout buffer, 10 µL of template RNA, 2 µL of RNase free water were brought to a 14 µL volume. Tubes were placed in a Veriti Thermal Cycler (Applied Biosystems) for 2 minutes at 42ºC and put directly on ice. 4 µL of 5x Quantiscript RT Buffer, 1 µL of RT Primer Mix, and 1 µL of Reverse-transcription master mix were added to the denatured RNA mix. The samples were placed again in the thermal cycler and run at 42 ºC for 30 minutes, and then 95 ºC for 5 minutes. The reverse transcription reaction products were diluted into 150 µL RNase free water and stored at -20 ºC until use for semi-quantitative real-time polymerase chain reaction. (qRT-PCR)

4.2.4.3 Semi-Quantitative Real-Time Polymerase Chain Reaction

Semi-quantitative real-time PCR (qRT-PCR) was conducted using the ViiA-7 Real Time PCR System (Applied Biosystems). Primers sequences were designed through an online tool and verified for specificity by using the Basic Local Alignment Search Tool (BLAST). Selected primers were synthesized at Integrated DNA Technologies (Coralville, Iowa, USA). The primers normalized to 100 µM in IDTE pH 8.0 or in Lab Ready format, were diluted in IDTE pH 8 to 50 nM. A list of primer sequences used is shown in Table 4.2.
The reactions were run in triplicate for at least 3 separate reactions on MicroAmp Optical 384-well plates (Applied Biosystems) and their amplifications were tracked by PowerUp SYBR Green fluorescent dye (Applied Biosystems). The total reaction volume was 10 µL. Completed plates were sealed and spun at 1200 rpm at 4 ºC for 5 minutes and then run in the ViiA-7 Real Time PCR system. The reaction conditions for stage one began with a hot start at 95 ºC to activate the polymerase. Stage 2 included 40 cycles of 95 ºC for 15 seconds then 60 ºC for 1 minute for annealing and extension. A melt curve was generated at 90 ºC for 15 seconds followed by at 60 ºC for 1 minute.
Results were analyzed using the comparative threshold (Ct) method and normalized relative to the quantification levels of the housekeeping gene RPL32. Fold change was calculated using the following formula:

\[ 2^{-\Delta \Delta Ct} \]

\[ \Delta \Delta Ct = \Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}} \]

\[ \Delta Ct_{\text{experimental}} = Ct_{\text{target}} - Ct_{\text{housekeeping}} \]

\[ \Delta Ct_{\text{control}} = Ct_{\text{calibrator (Day 0 50 nM FA)}} - Ct_{\text{housekeeping}} \]

Fold change was then compared between molar equivalent treatments (FA vs. 5MTHF) and within folate type (FA or 5MTHF).

4.2.5 Global DNA methylation Analysis

4.2.5.1 Genomic DNA Isolation:

Total genomic DNA was extracted using the QIAmp® DNA Mini DNA extraction and purification kit (Qiagen Cat. 51104). The final preparation had a ratio of A260:A280 between 1.8 and 2.0, indicating high quality and free of RNA and protein contamination. The concentration of each DNA sample was determined as the mean of three independent spectrophotometric readings. The DNA samples were stored at -20 ºC until further analysis.

4.2.5.2 Global DNA Methylation

The methylation of CpG sites in DNA was determined by the in vitro methyl acceptance assay using \[^{3}H\text{-methyl}]\text{SAM}\) as a methyl donor and a prokaryotic CpG DNMT, Ss1, as previously described (144, 145). Briefly, DNA, buffer, Ss1, and \[^{3}H\text{-methyl}]\text{SAM}\) was incubated for 1 hour at 30º C. the solution was dried under lamps on Whatman DAEA 81 filter paper. The dried filter paper was washed twice with washing buffer to wash off excess reagents, leaving only radio-labelled DNA on the filter paper. The filter papers were put into 10 mL scintillation
cocktail to resuspend the DNA in solution. The radioactivity of the solution was then measured. This assay results are reported as DPM (disintegration per minute) per 10 ng of DNA and is an inverse indicator of endogenous DNA methylation status. For example, the higher exogenous \[^{3}H\text{-methyl}\] incorporation, the lower endogenous DNA methylation status. All analyses were performed in quadruplicate using three different plates of cells.

4.3 Statistical analysis

For all data, comparison of means between groups and within groups were determined using 2-way ANOVA (growth curves, folate assay, DNA methylation n=3; qRT-PCR n=6) using GraphPad Prism 6 (California, USA) and corrected for multiple comparisons using Tukey’s test. Results are expressed as mean ± SD unless otherwise stated. All significance tests were two-sided and were considered significant at p-value < 0.05.

4.4 Results

4.4.1 FA vs. 5MTHF: cell growth curves

4.4.1.1 Caco-2 (refer to figure 4.1)

In cells grown in FA, there were no significant differences in cell growth among different FA concentrations. In contrast, in cells grown in 5MTHF, cells grown in 5 nM and 50 nM concentrations grew significantly slower than those grown in 500 nM and 1000 nM concentrations. When comparing between FA and 5MTHF at equimolar concentrations, there were no significant differences in growth at 5 nM and 50 nM concentrations. However, cells supplemented with 500 nM 5MTHF exhibited significantly faster growth rates than those supplemented with 500 nM FA on day 11 and day 15. In cells treated with 1000 nM folate, cells supplemented with 5MTHF had a significantly faster growth rate than those supplemented in FA only on day 15. HCT116 (Figure 4.2)
Due to the shorter doubling time of HCT116 cells, cells were split at more frequent intervals than for Caco-2 to avoid plateauing cell growth. In cells grown in FA, cells treated with 5 nM exhibited significantly slower growth rates than those treated with 50 nM, 500 nM and 1000 nM on day 14 and day 17. On day 17, cells treated with 50 nM FA had a significantly slower growth rate than those grown in 500nM and 1000 nM FA. In cells grown in 5MTHF, cells treated with 5 nM exhibited a significantly slower growth rate than cells grown in 50 nM, 500 nM and 1000 nM, while no differences were observed among cells grown in the latter 3 5MTHF concentration, on day 14. On day 17, cells treated by with 5 nM had a significantly slower growth rate than cells treated with 50, nM, 500 nM, and 1000 nM. On day 17, cells treated to 50 nM 5MTHF exhibited a significantly slower growth than cells supplemented with 1000 nM 5MTHF while no differences were observed between 50 nM and 500 nM and between 500 nM and 1000 nM. When comparing between FA and 5MTHF at equimolar concentrations, cells grown in 5 nM, 50 nM and 500 nM 5MTHF exhibited significantly faster growth rates than those grown in the corresponding concentrations of FA on day 14 and day 17. Interestingly, however, cells treated with 1000 nM 5MTHF had a significantly faster growth rate than those treated with 1000 nM FA only on day 17.
Figure 4.1 Caco-2 growth curves. Top two graphs represent comparison within same folate form A) FA B) 5MTHF. Bottom four graphs are comparisons between equimolar concentrations of FA and 5MTHF at C) 5 nM D) 50 nM E) 500 nM F) 1000 nM. Different letters represent statistical difference between folate concentrations of same folate form. * represents significant difference between FA vs. 5MTHF at equimolar supplemental concentrations (p<0.05) Results are presented as Means ± SD
Figure 4.2 HCT 116 growth curves Top two graphs represent comparison within same folate form A) FA B) 5MTHF. Bottom four graphs are comparisons between equimolar concentrations of FA and 5MTHF at C) 5 nM D) 50 nM E) 500 nM F) 1000 nM. Different letters represent statistical difference between folate concentrations of same folate form. * represents significant difference between FA vs. 5MTHF at equimolar supplemental concentrations (p<0.05) Results are presented as Means ± SD
4.4.2 FA vs. 5MTHF: intracellular folate concentrations on day 7 and day 14

4.4.2.1 Caco-2 (Refer to figure 4.3)

For cells grown in FA, significant differences in intracellular folate concentrations were observed among cells grown in different concentrations of FA on day 7 and day 14. Cells grown in 5 nM FA had significantly lower intracellular folate concentrations than cells grown in 50 nM, 500 nM and 1000 nM on day 7 and day 14. Cells grown in 50 nM FA had significantly higher intracellular folate concentrations than those grown in 5 nM FA and had significantly lower intracellular folate concentrations than those grown in 500 nM and 1000 nM FA on day 7 and day 14. There were no differences in intracellular folate concentrations between cells grown in 500 nM and 1000 nM FA on day 7 or day 14.

For cells grown in 5MTHF, cells grown in 5 nM and 50 nM 5MTHF had significantly lower intracellular folate concentrations than those grown in 500 nM and 1000 nM 5MTHF on day 7, while no differences were observed between 5 and 50 nM MTHF and between 500 and 1000 nM MTHF. On day 14, cells grown in 5 nM 5MTHF had significantly lower intracellular folate concentrations than those grown in 50 nM, 500 nM and 1000 nM 5MTHF. On day 14, cells grown in 50 nM 5MTHF had significantly lower intracellular folate concentrations than those grown in 500 nM and 1000 nM 5MTHF, while no differences were observed between 500 and 1000 nM 5MTHF.

When intracellular folate concentrations were compared between equimolar concentrations of FA and 5MTHF, no differences were observed between cells grown in the corresponding concentrations of FA and 5MTHF on day 7. However, on day 14, cells grown in 500 nM and 1000 nM 5MTHF had significantly higher intracellular folate concentrations than those grown in the corresponding concentrations of FA.
Figure 4.3 Caco-2 Intracellular folate concentrations Top two graphs represent comparison within same folate form A) FA B) 5MTHF. Bottom four graphs are comparisons between equimolar concentrations of FA and 5MTHF at C) 5 nM D) 50 nM E) 500 nM F) 1000 nM. Different letters represent statistical difference between folate concentrations of same folate form. * represents significant difference between FA vs. 5MTHF at equimolar supplemental concentrations (p<0.05) Results are presented as Means ± SD

4.4.2.2 HCT 116 (Refer to figure 4.4)

For cells grown in FA, cells grown in 5 nM, 50 nM and 1000 nM had significantly lower intracellular folate concentrations than those grown in 500 nM FA on day 7, while no differences were observed among these 3 FA concentrations. On day 14, cells grown in 5 nM and 50 nM FA had significantly lower intracellular folate concentrations than those grown in 1000 nM FA. Also, on day 14, cells grown in 5 nM FA had significantly lower intracellular folate concentrations.
than those grown in 500 nM FA. No differences were observed between cells grown in 50 and 500 nM FA and between those grown in 500 and 1000 nM on day 14.

For cells grown in 5MTHF, those grown in 5 nM and 50 nM 5MTHF had significantly lower intracellular folate concentrations than those grown in 500 and 1000 nM 5MTHF on both day 7 and day 14, while no differences were observed between those grown in 5 and 50 nM 5MTHF. Cells grown in 500 nM 5MTHF had significantly lower intracellular folate concentrations than those grown in 1000 nM 5MTHF on both day 7 and day 14.

When intracellular folate concentrations were compared between FA and 5MTHF at equimolar concentrations, no differences were observed between cells grown in 5 nM FA and 5 nM 5MTHF and between those grown in 50 nM FA and 50 nM 5MTHF. However, cells grown in 500 nM MTHF had significantly higher intracellular folate concentrations than those grown in 500 nM FA on day 14 but not on day 7. Cells grown in 1000 nM 5MTHF had significantly higher intracellular folate concentrations than those grown in 1000 nM FA on both day 7 and day 14.
Figure 4.4 HCT116 Intracellular folate concentrations Top two graphs represent comparison within same folate form A) FA B) 5MTHF. Bottom four graphs are comparisons between equimolar concentrations of FA and 5MTHF at C) 5 nM D) 50 nM E) 500 nM F) 1000 nM. Different letters represent statistical difference between folate concentrations of same folate form. * represents significant difference between FA vs. 5MTHF at equimolar supplemental concentrations (p<0.05) Results are presented as Means ± SD

4.4.3 Comparative Supplemental Effect on mRNA expression via qRT-PCR at day 14

Below are results for relative mRNA expression normalized to cells from Day 0 for Caco-2 and HCT116 cells. Results will be summarized in the following groupings: intracellular folate
uptake/transport (*GCPII, FR, RFC, PCFT*), intracellular folate retention and hydrolysis (*FPGS, GGH*), intracellular folate metabolism and DNA synthesis (*DHFR, SHMT, TS*), methionine cycle (*MTR, MTRR, MTHFR*), and DNA methylation (*MBD2, DNMT1, DNMT3A, and DNMT3B*).

Gene expression in Caco-2 will be summarized first followed by HCT116.

4.4.3.1 Caco-2

**Folate Uptake/Transport (refer to figure 4.5):**

In Caco-2 cells grown in FA, *GCPII* mRNA expression generally increased from 5 nM up to 500 nM followed by a decrease to the level observed with 5nM at 1000 nM FA. *GCPII* mRNA expression was significantly higher in cells grown in 500 nM FA than in cells grown in 5 nM and 1000 nM FA, while no difference was observed between 50 and 500 nM FA. In cells grown in 5MTHF, *GCPII* mRNA expression was significantly lower in cells grown in 1000 nM 5MTHF than in those grown in 5 nM, 50 nM and 500 nM 5MTHF, while no differences were observed among these latter 5MTHF concentrations. No differences in *GCPII* mRNA expression were observed between FA and 5MTHF at any of the 4 equimolar concentrations of FA and 5MTHF.

The steady-state mRNA expression levels of the *FR* gene increased as supplemental concentrations of FA increased *FR* mRNA expression was significantly higher in cells supplemented with 500 nM FA or 1000 nM FA than in cells supplemented with 5 nM or 50 nM FA; no differences were observed between 5 nM and 50 nM FA and between 500 nM and 1000 nM. In contrast, no differences in *FR* mRNA expression was noted among cells grown in 4 different 5MTHF concentrations. Furthermore, no differences in *FR* mRNA expression between cells grown in equimolar concentrations FA and 5MTHF.
PCFT mRNA expression was significantly higher in cells grown at 50 nM FA than in those grown in 5 nM FA, 500 nM FA, and 1000 nM, while no differences were observed among these latter 3 FA concentrations. In cells grown in 5MTHF, PCFT mRNA expression was significantly higher in those grown in 5 nM 5MTHF than in those grown in 50, 500, and 1000 nM 5MTHF concentrations. PCFT mRNA expression was significantly higher in cells treated with 5 nM or 500 nM 5MTHF than in 5 nM or 500 nM FA, respectively. However, no differences were observed between cells grown in equimolar concentrations of 50 nM FA and 5MTHF or between cells grown in equimolar concentrations of 1000 nM FA and 5MTHF.

RFC mRNA expression was significantly lower in cells grown in 1000 nM FA than in those grown in 5 nM, 50 nM, and 500 nM. In cells grown in 5MTHF, RFC mRNA expression was significantly higher in 5 nM and 500 nM compared to 50 nM and 1000 nM, while no differences were observed between 5 and 500 nM and between 50 and 1000 nM. Between folate forms, RFC mRNA expression was higher in 5 nM and 500 nM 5MTHF compared to the corresponding equimolar FA concentrations. However, no differences were observed between 50 nM FA and 50 nM 5MTHF or between 1000 nM FA and 1000 nM 5MTHF.
**Figure 4.5 Caco-2 Folate Uptake/Transport** Comparative supplemental effect on mRNA expression. Fold changes were normalized to Day 0. Bars represent means with 95% confidence intervals. Groups with different letters represent significant differences within FA concentrations. Groups with different numbers represent significant differences among 5MTHF concentrations. Asterisks (*) represent differences between FA and 5MTHF at equimolar concentrations (n=6). Significance was determined if p<0.05.

**Intracellular Folate Retention and Hydrolysis and DHFR (refer to figure 4.6):**

In cells grown in FA, *FPGS* mRNA expression decreased as FA concentrations in the media increased in a dose-responsive manner. However, no significant difference was observed between cells grown in 500 nM FA and those grown in 1000 nM FA. In cells grown in 5MTHF, cells grown in 500 nM had significantly higher *FPGS* mRNA expression compared to those grown in 50 nM and 1000 nM, while no significant difference was observed between these two 5MTHF concentrations. Cells grown in 5 nM 5MTHF had *FPGS* mRNA expression level intermediate between that associated with 50 nM and 1000 nM 5MTHF and that associated with
500 nM 5MTHF. When comparing between equimolar FA and 5MTHF concentrations, FPGS mRNA expression was significantly higher in cells grown in FA than in those grown in 5MTHF at 5 nM and 50 nM. At 500 nM, however, the reverse occurred and cells grown in 5MTHF had higher FPGS mRNA expression levels than those grown in FA. At 1000 nM, no significant difference was observed between cells grown in FA and those grown in 5MTHF.

GGH mRNA expression was significantly higher in cells grown in 5 nM FA than those grown in 50, 500, and 1000 nM FA, while no significant differences were observed among these latter 3 FA concentrations. No significant differences observed in GGH mRNA expression among cells grown in 4 different 5MTHF concentrations. When comparing equimolar concentrations of FA and 5MTHF, GGH mRNA expression was significantly higher in cells grown at 5 nM FA than in cells grown in 5 nM 5MTHF. However, no significant differences in GGH mRNA expression were observed between FA and 5MTHF at 50, 500, and 1000 nM.

Figure 4.6 Caco-2 Folate glutamate hydrolase and synthase Comparative supplemental effect on mRNA expression: mRNA expression levels. Fold changes were normalized to Day 0 (50 nM FA acclimatized). Bars represent means with 95% confidence intervals. Groups with different letters represent significant differences within FA concentrations. Groups with different numbers represent significant differences within 5MTHF concentrations. Asterisks (*) represent differences between FA and 5MTHF at equimolar concentrations
Intracellular folate metabolism and DNA Synthesis (*refer to figure 4.7*):

No significant differences in *DHFR* mRNA expression were observed among cells grown in different concentrations of FA or 5MTHF. Furthermore, no significant differences in *DHFR* mRNA expression between cells grown in 4 equimolar concentrations of FA and 5MTHF.

*SHMT* mRNA expression was significantly higher in cells grown in 50 nM FA than those grown in 5 nM FA. *SHMT* mRNA expression in cells grown in 500 nM and 1000 nM FA were between that of cells grown in 5 nM FA and 50 nM FA. *SHMT* mRNA expression in cells grown in 50 nM and 1000 nM was significantly higher than those grown in 5 nM and 500 nM. No significant differences were observed between cells grown in 5 nM and 500 nM 5MTHF and between cells grown in 50 nM and 1000 nM 5MTHF. When comparing between equimolar FA and 5MTHF concentrations, *SHMT* mRNA expression was significantly higher in cells supplemented with 1000 nM 5MTHF than those grown in 1000 nM FA, while no differences were observed at the 3 lower equimolar FA and 5MTHF concentrations.

Cells grown 5 nM FA had significantly lower *TS* mRNA expression than cells grown at the 3 higher FA concentrations. Cells grown in 50 nM had significantly higher *TS* mRNA expression than cells grown in 500 and 1000 nM FA, while no significant difference was observed between 500 and 1000 nM FA. In cells grown in 5MTHF, *TS* mRNA expression levels generally rose with increasing concentrations of 5MTHF. Cells grown in 5 nM 5MTHF had significantly lower *TS* mRNA expression than those grown in 50 nM and 1000 nM 5MTHF, while those grown in 1000 nM 5MTHF had significantly higher *TS* mRNA expression than those grown in 50 nM and 500 nM 5MTHF. No significant differences in *TS* mRNA expression were observed between cells grown in 5 nM and 500 nM and between cells grown in 50 nM and 500 nM 5MTHF. When comparing equimolar concentrations of FA and 5MTHF, *TS* mRNA expression was significantly
higher in cells grown in 50 nM and 500 nM than those grown in the corresponding equimolar concentrations of 5MTHF. However, no significant differences in TS mRNA expression were observed in cells grown in 5 nM and 1000 nM FA compared with those grown in the equimolar concentrations of 5MTHF.

Figure 4.7 Caco-2 Intracellular folate metabolism and DNA synthesis: Comparative supplemental effect on mRNA expression. Fold changes were normalized to Day 0 (50 nM FA acclimatized). Bars represent means with 95% confidence intervals. Groups with different letters represent significant differences within FA concentrations. Groups with different numbers represent significant differences within 5MTHF concentrations. Asterisks (*) represent differences between FA and 5MTHF at equimolar concentrations (n=6) Significance was determined if p<0.05.

Methionine Cycle (refer to figure 4.8):

MTR mRNA expression was not significantly different among cells grown in 4 concentrations of FA. MTR mRNA expression was significantly higher in cells grown in 500 nM
5MTHF than those grown in 5 nM, 50 nM, and 1000 nM 5MTHF, while no significant differences were observed among the latter 3 5MTHF concentrations. When comparing equimolar concentrations of FA and 5MTHF, MTR mRNA expression was higher in cells grown in 500 nM FA than those grown in 500 nM 5MTHF, while no significant differences were observed at other 3 equimolar concentrations of FA and 5MTHF.

No significant differences in MTRR mRNA expression were observed among the 4 increasing concentrations of FA or 5MTHF. However, MTRR mRNA expression was significantly higher in cells grown in 5MTHF than in those grown in FA at all equimolar concentrations.

No differences in MTHFR mRNA expression was observed among the 4 increasing FA concentrations. MTHFR mRNA expression was significantly lower in cells grown in 1000 nM 5MTHF than in cells grown in 5nM and 500 nM 5MTHF; however, no difference was observed between cells grown in 50 nM and those grown in 1000 nM 5MTHF. Furthermore, no significant differences were observed among cells grown in 5 nM, 50 nM, and 500 nM 5MTHF. Cells grown in 5MTHF had significantly higher MTHFR mRNA expression compared to those grown in FA at 5 nM, 500 nM and 1000 nM equimolar concentrations, but not at 50 nM equimolar concentration.
**Figure 4.8 Caco-2 Methionine Cycle**: Comparative supplemental effect on mRNA expression. Fold changes were normalized to Day 0 (50 nM FA acclimatized). Bars represent means with 95% confidence intervals. Groups with different letters represent significant differences within FA concentrations. Groups with different numbers represent significant differences within 5MTHF concentrations. Asterisks (*) represent differences between FA and 5MTHF at equimolar concentrations (n=6) Significance was determined if p<0.05.

DNA Methylation (*refer to figure 4.9*):

No significant differences in *MBD2* mRNA expression levels were observed among cells grown in 4 increasing FA concentrations. *MBD2* mRNA expression was significantly higher in cells grown in 5 nM 5MTHF compared with those grown in 50 nM and 500 nM 5MTHF. Cells grown in 1000 nM 5MTHF had *MBD2* mRNA expression intermediate between 5 nM and 50 and 500 nM 5MTHF. *MBD2* mRNA expression was significantly higher in cells grown in 5 nM 5MTHF than in those grown in 5 nM FA. However, no significant differences in *MBD2* mRNA expression between cells grown in other 3 higher equimolar concentrations of FA and 5MTHF.
There were no differences in \textit{DNMT1} mRNA expression levels in cells treated with different concentrations of FA. In cells treated with 5MTHF, 5 nM and 500 nM supplementation resulted in significantly higher \textit{DNMT1} mRNA expression compared to 50 nM and 1000 nM. Between equimolar concentrations of FA and 5MTHF, \textit{DNMT1} mRNA expression was significantly higher in cells supplemented with 5 nM, 50 nM and 500 nM of 5MTHF.

Cells supplemented with FA had significantly higher \textit{DNMT3a} mRNA expression in 5 nM than in 1000 nM. Cells supplemented with 500 nM 5MTHF had higher \textit{DNMT3a} expression at 500 nM compared to 50 nM and 1000 nM. Cells supplemented with 5MTHF had higher \textit{DNMT3a} expression compared to FA at 5 nM, 500 nM and 1000 nM concentrations.

Cells supplemented with 500 nM FA had significantly higher \textit{DNMT3b} expression than cells supplemented with 5 nM FA and 1000 nM FA. For all equimolar concentrations, 5MTHF had higher DNMT3b expression than FA.
Figure 4.9 Caco-2 DNA methylation: Comparative supplemental effect on mRNA expression. Fold changes were normalized to Day 0. Bars represent means with 95% confidence intervals. Groups with different letters represent significant differences within FA concentrations. Groups with different numbers represent significant differences within 5MTHF concentrations. Asterisks (*) represent differences between FA and 5MTHF at equimolar concentrations (n=6). Significance was determined if p<0.05.
Table 4.3 General summary of mRNA expression in Caco-2 with increasing dose and response to folate form

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<th>Gene</th>
<th>Dose effects</th>
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<td>DNMT3b</td>
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General summary of comparative mRNA expression. Dose effects refer to the presence of significant differences between concentrations within the same folate form. ↑ is used if expression was significantly greater at higher levels of supplementation compared to lower levels of supplementation. ↓ is used if expression was significantly lower at higher levels of supplementation compared to lower levels of supplementation. FA vs. 5MTHF expression levels are compared at [low]: 5 nM and 50 nM and [high] 500 nM and 1000 nM doses. If significant differences were observed, the folate form with the higher expression level is stated.

4.4.3.2 HCT 116

Folate transporters (refer to figure 4.10):

 FR mRNA expression was significantly higher in cells supplemented at 500 nM FA compared to 5 nM FA and 50 nM FA. This was also true for cells supplemented with 5MTHF. At 5 nM 5MTHF and 500 nM 5MTHF supplementation resulted in significantly greater expression compared to equimolar FA supplementation.

 PCFT expression was significantly higher at 5 nM FA compared to all other FA concentrations. There were no differences in PCFT expression levels between 5MTHF concentrations. PCFT expression was significantly higher in 5 nM FA than 5 nM 5MTHF. At
500 nM, 5MTHF supplementation resulted in significantly higher \textit{PCFT} mRNA expression compared to equimolar FA supplementation.

FA supplementation elicited a dose response for \textit{RFC} mRNA expression. \textit{RFC} expression was significantly higher in 5nM FA than all other FA concentrations (5 nM FA > 50 nM FA, > 500 nM FA). 5 nM 5MTHF supplemented cells had lower RFC expression than 500 nM 5MTHF. In 5 nM and 1000 nM, FA had higher \textit{RFC} expression than 5MTHF. In 500 nM, FA had lower expression than 5MTHF.

In FA supplemented cells, \textit{GCPII} mRNA expression did not differ between the 4 supplemental levels. In 5MTHF supplemented cells, supplementation with 500 nM 5MTHF resulted in significantly higher \textit{GCPII} mRNA expression levels compared to 5 nM 5MTHF. There were no significant differences between folate forms.
**Figure 4.10 HCT116 Folate uptake and transport:** Comparative supplemental effect on mRNA expression: mRNA expression levels. Fold changes were normalized to Day 0 (50 nM FA acclimatized). Graphs represent means with 95% confidence intervals. FA is represented by letters and 5MTHF is represented by numbers. Different letters represent significant differences between FA concentrations. Different numbers represent significant differences between 5MTHF concentrations. Differences between equimolar concentrations are represented by *.

Significance was determined if p<0.05

**Glutamate hydrolase and synthase (refer to figure 4.11):**

*FPGS* expression was significantly lower at 500 nM FA compared to 5 nM and 1000 nM FA. *FPGS* mRNA expression was significantly higher with 500 nM 5MTHF supplementation compared to all other supplemental 5MTHF concentrations. Compared to 5MTHF, FA had higher *FPGS* mRNA expression at 5nM and 1000 nM. Compared to 5MTHF, FA had lower *FPGS* mRNA expression at 500 nM.
GGH expression was significantly higher in cells supplemented with 500 nM FA compared to all other concentrations of FA. This was also true for cells supplemented with 5MTHF. There were no differences in mRNA expression between equimolar folate concentrations (FA vs. 5MTHF).

Figure 4.11 HCT116 Folate glutamate hydrolase and synthase: Comparative supplemental effect on mRNA expression: mRNA expression levels. Fold changes were normalized to Day 0. Graphs represent means with 95% confidence intervals. FA is represented by letters and 5MTHF is represented by numbers. Different letters represent significant differences between FA concentrations. Different numbers represent significant differences between 5MTHF concentrations. Differences between equimolar concentrations are represented by *. (n=6) Significance was determined if p<0.05

DNA Synthesis (refer to figure 4.12):

DHFR was expressed significantly higher at 5 nM FA compared to all other FA treatments. DHFR mRNA was expressed significantly higher at 500 nM 5MTHF compared to all other 5MTHF concentrations. FA had higher DHFR expression compared to 5MTHF at 5 nM and 500 nM concentrations.

SHMT had significantly lower mRNA expression levels at 500 nM FA compared to all other FA concentrations. Cells supplemented with 5 nM 5MTHF expressed SHMT mRNA
significantly less than cells supplemented with 1000 nM SHMT. Between FA and 5MTHF, 5 nM FA had higher SHMT expression than 5 nM 5MTHF.

TS was expressed significantly higher at 5 nM compared to all other folate concentrations for both FA and 5MTHF. Expression was also higher in 1000 nM compared to 500 nM for cells treated with FA and 5MTHF. TS expression was significantly higher in cells treated with 5 nM FA compared to 5 nM 5MTHF.

**Figure 4.12 HCT116 DNA synthesis:** Comparative supplemental effect on mRNA expression: mRNA expression levels. Fold changes were normalized to Day 0 (50 nM FA acclimatized). Graphs represent means with 95% confidence intervals. FA is represented by letters and 5MTHF is represented by numbers. Different letters represent significant differences between FA concentrations. Different numbers represent significant differences between 5MTHF concentrations. Differences between equimolar concentrations are represented by *. (n=6) Significance was determined if p <0.05

**Methionine Cycle (refer to figure 4.13):**

MTR mRNA expression was significantly higher in cells treated with 5 nM FA compared to all other FA concentrations. MTR mRNA expression was significantly higher in cells supplemented with 5 nM 5MTHF compared to 50 nM and 1000 nM 5MTHF. MTR expression was significantly lower at 50 nM 5MTHF compared to 500 nM and 1000 nM.
5MTHF, 5MTHF had higher expression of *MTR* than FA at 5 nM, 500 nM and 1000 nM concentrations.

*MTRR* expression was significantly lower in cells grown in 1000 nM FA compared to 5 nM and 50 nM FA concentrations. 500 nM FA also expressed lower *MTRR* than 5 nM FA. Expression was lowest in 50 nM 5MTHF treated cells compared to all other 5MTHF concentrations. Supplementation with 5MTHF resulted in significantly higher MTRR mRNA expression at 500 nM and 1000 nM compared to cells supplemented with equimolar FA. Cells supplemented with 50 nM FA expressed *MTRR* mRNA significantly more than cells supplemented with 50 nM 5MTHF.

There were no differences in *MTHFR* mRNA expression at different concentrations of FA. *MTHFR* expression was significantly lower in 50 nM 5MTHF supplemented cells than 500 nM and 1000 nM 5MTHF concentrations. There were no differences in *MTHFR* mRNA expression between equimolar concentrations of FA and 5MTHF.

**Figure 4.13** HCT116 Methionine cycle: Comparative supplemental effect on mRNA expression: mRNA expression levels. Fold changes were normalized to Day 0 Graphs represent means with 95% confidence intervals. FA is represented by letters and 5MTHF is represented by numbers. Different letters represent significant differences between FA concentrations. Different numbers represent significant differences between 5MTHF concentrations. Differences between equimolar concentrations are represented by *. (n=6) Significance was determined if p <0.05.
DNA Methylation (refer to figure 4.14)

In cells supplemented with FA, there were no significant differences in *MBD2* expression in cells between all 4 concentrations of FA. In cells supplemented with 5MTHF, 500 nM concentrations resulted in significantly higher *MBD2* mRNA expression levels compared to the 3 other concentrations of 5MTHF. Between equimolar folate concentrations, cells supplemented with 500 nM 5MTHF had higher *MBD2* mRNA expression levels compared to cells supplemented with equimolar FA.

In cells supplemented with FA, *DNMT1* expression was significantly higher in 5 nM FA compared the 3 other FA concentrations. *DNMT1* expression was significantly lower in cells supplemented with 1000 nM 5MTHF than 50 nM and 500 nM 5MTHF. When supplemented with 5MTHF, DNMT1 mRNA expression was highest at 500 nM. Between equimolar concentrations of 5MTHF and FA, *DNMT1* expression was significantly higher in 5 nM FA, 50 nM 5MTHF, and 500 nM 5MTHF compared to their equimolar counterparts.

*DNMT3a* expression was significantly lower in 500 nM FA compared to 5 nM FA. *DNMT3a* mRNA expression was the highest in cells supplemented with 500 nM 5MTHF compared to cells supplemented with 5 nM and 1000 nM 5MTHF. FA and 5MTHF appear to take on opposing trends in DNMT3A expression. While expression increases and then decrease for 5MTHF, expression decreases then increases for FA. There were significant differences between FA and 5MTHF at 500 nM supplementation.

The opposing trend seen with *DNMT3b* is also seen in *DNMT3b*. 500 nM 5MTHF supplementation resulted in significantly higher *DNMT3b* mRNA expression compared to all other 5MTHF concentrations. 5 nM FA supplementation resulted in significantly higher
DNMT3b expression levels compared to 50 nM and 500 nM FA supplementation. When comparing equimolar folate concentrations, 5nM FA, 500 nM 5MTHF, and 1000 nM FA supplementation resulted in higher DNMT3b compared to their equimolar counterpart.

For all genes involved with DNA methylation a trend is observed. Expression levels increase and then drop at 1000 nM for 5MTHF while expression levels generally decrease when supplemented with FA.

**Figure 4.14 HCT116 DNA methylation:** Comparative supplemental effect on mRNA expression: mRNA expression levels. Fold changes were normalized to Day 0 (50 nM FA acclimatized). Graphs represent means and 95% confidence intervals. FA is represented by letters and 5MTHF is represented by numbers. Groups with different notations are considered different. Differences between equimolar concentrations are represented by *. Significance was determined at p<0.05.
Table 4.4 General summary of mRNA expression in HCT116 with increasing dose and response to folate form

<table>
<thead>
<tr>
<th>Gene</th>
<th>FA vs. 5MTHF</th>
<th>Dose effects</th>
<th>FA</th>
<th>5MTHF</th>
<th>[low folate]</th>
<th>[high folate]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCPH</td>
<td>--</td>
<td>↑</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>FRA</td>
<td>↑</td>
<td>↑↓</td>
<td>5MTHF</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PCHF</td>
<td>↓↑</td>
<td>--</td>
<td>FA</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>RFC</td>
<td>↓↑</td>
<td>↑↓</td>
<td>FA</td>
<td>500 nM 5MTHF</td>
<td>1000 nM FA</td>
<td>--</td>
</tr>
<tr>
<td>FPGS</td>
<td>↑↑</td>
<td>↑↓</td>
<td>FA</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GGH</td>
<td>↓↑↑</td>
<td>↑↓</td>
<td>FA</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DHFR</td>
<td>↓</td>
<td>↑↓</td>
<td>FA</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SHMT</td>
<td>↑</td>
<td>↑</td>
<td>FA</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TS</td>
<td>↑</td>
<td>↑↑</td>
<td>FA</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MTR</td>
<td>↓</td>
<td>↑↑</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MTRR</td>
<td>--</td>
<td>↑</td>
<td>FA</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MTHFR</td>
<td>--</td>
<td>--</td>
<td>FA</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MBT2</td>
<td>--</td>
<td>↑↓</td>
<td>--</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DNMT1</td>
<td>↓</td>
<td>↑</td>
<td>5nM FA; 50 nM 5MTHF</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DNMT3a</td>
<td>↓</td>
<td>↑</td>
<td>--</td>
<td>5MTHF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DNMT3b</td>
<td>↓</td>
<td>↑</td>
<td>FA</td>
<td>500 nM 5MTHF</td>
<td>1000 nM FA</td>
<td>--</td>
</tr>
</tbody>
</table>

General summary of comparative mRNA expression. Dose effects refer to the presence of significant differences between concentrations within the same folate form. ↑ is used if expression was significantly greater at higher levels of supplementation compared to lower levels of supplementation. ↓ is used if expression was significantly lower at higher levels of supplementation compared to lower levels of supplementation. FA vs. 5MTHF expression levels are compared at [low]: 5 nM and 50 nM and [high] 500 nM and 1000 nM doses. If significant differences were observed, the folate form with the higher expression level is stated.

4.4.4 FA vs. 5MTHF: comparative global DNA methylation on day 7 and day 14

Below are global DNA methylation results for Caco-2 and HCT116 as dpm/10 ng DNA (an inverse indicator of global DNA methylation). A dose dependent response for both cell lines are observed. However, cell lines take on opposing trends. DNA of Caco-2 cells supplemented with FA were generally hypomethylated compared to cells supplemented with 5MTHF. The opposite was true for HCT116 cells. Cells supplemented with FA resulted in higher global DNA methylation compared to cells supplemented with 5MTHF.

4.4.4.1 Caco-2 (refer to figure 4.15)

Significant differences in global DNA methylation were observed at either day 7 or day 14.
At day 7:

1000 nM FA supplementation resulted in significantly higher DNA methylation compared to all other concentrations. 5 nM and 50 nM FA supplemented cells had higher global DNA methylation compared to cells supplemented with 500 nM FA. Interestingly, a clear binary response is seen in cells supplemented with 5MTHF. 500 nM and 1000 nM 5 MTHF supplementation had significantly higher global DNA methylation compared to cells supplemented with 5 nM and 50 nM 5MTHF.

Between equimolar concentrations of FA and 5MTHF, supplementation with 500 nM and 1000 nM 5MTHF resulted in significantly greater DNA methylation.

At day 14:

A clear dose response is seen in global DNA methylation patterns of cells supplemented with FA. 500 nM FA supplementation resulted in higher global DNA methylation compared to 1000 nM FA. 1000 nM FA supplemented cells had significantly higher global DNA methylation compared to 50 nM FA supplemented cells. 50 nM FA supplemented cells had significantly higher global DNA methylation compared to cells supplemented with 5 nM FA. In cells supplemented with 5MTHF, 500 nM 5MTHF supplemented resulted in significantly greater global DNA methylation compared to all other 5MTHF supplemental concentrations.

Global DNA methylation was significantly greater in cells supplemented with 5 nM and 50 nM 5MTHF compared to equimolar FA supplementation.
Figure 4.15 Caco-2 global DNA methylation: DPM is an inverse indicator of global DNA methylation. Higher DPM means less global DNA methylation. Top two graphs represent comparison within same folate form A) FA B) 5MTHF. Bottom four graphs are comparisons between equimolar concentrations of FA and 5MTHF at C) 5 nM D) 50 nM E) 500 nM F) 1000 nM. Different letters represent statistical difference between folate concentrations of same folate form. * represents significant difference between FA vs. 5MTHF at equimolar supplemental concentrations (p<0.05) Results are presented as Means ± SD

4.4.4.2 HCT116 (refer to figure 4.16)

FA supplementation did not affect global DNA methylation levels. However, 5MTHF supplementation did affect global DNA methylation levels. At day 7, cells supplemented with 5 nM 5MTHF had significantly less global DNA methylation compared to cells supplemented with
all other 5MTHF concentrations. 1000 nM 5MTHF supplemented cells had significantly less DNA methylation compared to 50 nM and 500 nM 5MTHF supplemented cells. At day 14, cells supplemented with 1000 nM 5MTHF had significantly less global DNA methylation compared to cells supplemented with all other 5MTHF concentrations. 5 nM 5MTHF supplemented cells had significantly less global DNA methylation than cells supplemented with 50 nM and 500 nM 5MTHF.

Comparatively between equimolar concentrations of FA and 5MTHF, cells supplemented with 5 nM and 50 nM FA had higher global DNA methylation compared to cells supplemented with equimolar 5MTHF at both day 7 and day 14.
**Figure 4.16 HCT116 global DNA methylation:** DPM is an inverse indicator of global DNA methylation. Higher DPM means less global DNA methylation. Top two graphs represent comparison within same folate form A) FA B) 5MTHF. Bottom four graphs are comparisons between equimolar concentrations of FA and 5MTHF at C) 5 nM D) 50 nM E) 500 nM F) 1000 nM. Different letters represent statistical difference between folate concentrations of same folate form. * represents significant difference between FA vs. 5MTHF at equimolar supplemental concentrations (p<0.05) Results are presented as Means ± SD
4.5 Discussion

Using an in vitro model of folate deficiency and supplementation, studies were undertaken to examine whether intracellular folate uptake/transport and metabolism and one-carbon transfer reactions involved in nucleotide biosynthesis and DNA methylation may respond differently to FA and 5MTHF. This is an important issue as FA supplementation has been purported to be associated with adverse health outcomes associated with high folate status and 5MTHF has been promoted as a safer alternative to FA in raising folate status. 5MTHF is as efficacious as FA in treating folate deficiency. However, it is unknown how 5MTHF compares to FA with at levels above sufficiency. We chose two human colon adenocarcinoma cell lines as a model to study this issue. These 2 cell lines have previously been used extensively to investigate the effects of folate deficiency in our lab as well as other labs (110, 114). These colon cancer cell lines are unique in 2 ways. Upon differentiation, Caco-2 forms tight junctions mimicking enterocyte morphology and has been used in many absorption studies for this reason (137). HCT116 has been previously genotyped and found to be heterozygous for the MTHFR polymorphism (138).

We demonstrated that FA and 5MTHF differentially affected cell growth; intracellular folate concentrations; mRNA expression of genes involved in folate uptake/transport and metabolism and one-carbon transfer reactions; and global DNA methylation in a cell-specific manner (refer to table 4.5). Overall, it was observed that 5MTHF supplementation compared to FA supplementation resulted in increased cellular proliferation, higher intracellular folate concentrations, and higher mRNA expression of folate transporters and DNA methylation proteins. Interestingly, 5MTHF supplementation in Caco-2 cells resulted in greater global DNA methylation compared to FA supplementation. However, these effects were opposite in HCT116.
Generally, clear dose-dependent responses were not always observed; however, folate form did have significant effects on all measures. This alludes to important implications in recommendations for folate supplementation. Below, I will discuss these findings, with a focus on folate form, in three sections: 1) Intracellular folate transport, retention, and hydrolysis; 2) cell growth and intracellular metabolism and; 3) DNA methylation.

Table 4.5: Generalized summary of findings: FA vs. 5MTHF

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Caco-2</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell proliferation</td>
<td>Increased with 5MTHF</td>
<td>Increased with 5MTHF</td>
</tr>
<tr>
<td>[Intracellular folate]</td>
<td>Increased with 5MTHF</td>
<td>Increased with 5MTHF</td>
</tr>
<tr>
<td>intracellular folate transport, retention, and hydrolysis:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FR, PCFT, RFC, GCPII, GGH, FPGS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular folate metabolism</td>
<td>Higher with FA at low doses</td>
<td>Non consistent; higher with FA at lower levels</td>
</tr>
<tr>
<td>Intracellular folate metabolism</td>
<td>Higher with 5MTHF at high doses</td>
<td></td>
</tr>
<tr>
<td>DNA synthesis</td>
<td>Only TS expression higher with FA</td>
<td>Higher with FA at lower levels; no difference at higher levels</td>
</tr>
<tr>
<td>DNA synthesis</td>
<td>Higher with 5MTHF</td>
<td></td>
</tr>
<tr>
<td>Methionine Cycle</td>
<td>Higher with 5MTHF</td>
<td></td>
</tr>
<tr>
<td>Methionine Cycle</td>
<td>Higher with 5MTHF</td>
<td></td>
</tr>
<tr>
<td>DNA methylation</td>
<td>Higher with 5MTHF</td>
<td>Inconsistent with dose</td>
</tr>
<tr>
<td>Global DNA methylation</td>
<td>Higher with 5MTHF</td>
<td>Higher with FA</td>
</tr>
</tbody>
</table>

Generalized finding focusing on the comparative differences between equimolar doses of FA and 5MTHF

4.5.1 FA vs. 5MTHF: intracellular folate transport, retention, and hydrolysis:

FR, PCFT, RFC, GCPII, GGH, FPGS

Based on previous studies, it was hypothesized that mRNA expression of genes involved in folate uptake and transport would decrease with increasing supplemental levels of both FA and 5MTHF. It was also hypothesized that folate form would not have any differences on mRNA expression. Overall, while inconsistent, some dose effects between FA and 5MTHFs supplementation were observed. Contrary to my hypothesis, folate form did have an effect on mRNA expression.
In Caco-2 cells, increasing FA supplementation resulted in significant expression differences in 4 of the 6 genes. Increasing supplemental levels of FA generally increased \( FR\alpha \) mRNA expression while decreasing \( RFC, GGH \) and \( FPGS \) mRNA. With increasing 5MTHF supplementation, there were no discernable patterns of expression even though there were significant differences between doses. Our a priori hypothesis was that mRNA expression of genes involved in folate uptake and transport would decrease with increasing supplemental levels of FA.

In HCT116 cells, there were fewer discernable patterns of expression for increasing supplementation for both FA and 5MTHF. When supplemented with increasing FA, HCT116 cells increased \( FR \) expression and decreased \( PCFT \) and \( RFC \) expression. 5MTHF supplementation had no discernable patterns except that expression of \( FR, RFC \), and \( FPGS \) was highest with 500 nM supplementation. Comparing between FA and 5MTHF supplementation, typically at low doses (5 nM) mRNA expression of these genes were higher in cells supplemented with FA and mRNA expression higher in cells supplemented at higher doses (500 nM) of 5MTHF. However, at 1000 nM, FA supplementation resulted in higher expression. This may allude to an adaptive response. HCT116 is heterozygous for \( MTHFR \) and may upregulate folate uptake at low levels of FA due to insufficient FA reduction and then at high FA concentrations due to pseudo-MTHFR deficiency as alluded to by Christiensen et al (139).

In contrast to our observations, evidence from the literature shows an inverse correlation between \( FR\alpha \) mRNA expression and extracellular folate concentrations (140). Most of the studies that examined the relationship between extracellular FA concentrations and \( FR\alpha \) mRNA expression were conducted in rodent or \textit{in vitro} models and assessed the relationship in different tissues (the majority being blood, lung, kidney, liver, head, and neck). Efficiency of folate
transport via folate transporters depends on tissue and pH. Our experiments may have shown different trends due to varying assay specificity. The expression of FR isoforms differs depending on tissue. The α isoform is mainly found on epithelial cells and the β isoform is found mainly in hematopoietic cells (140). They are found tandem in genomic DNA and have 71% identity in their amino acid form (140). Particularly in in vivo studies where tissues consist of a heterogeneous population of cells, it is possible to mistaken the mRNA or protein expression of both isoforms as just one or the other if the designed primers and antibodies are not specific for the target isoform.

It is also difficult to compare results of many of these studies because previous studies employed different folate concentrations and folate types. The inconsistency of tested conditions may account for the varying conclusions made by each study. Ashokkumar et al. (114) observed that FA ‘oversupplementation’ resulted in a downregulation of RFC, FR, and PCFT in Caco-2 and HK-2 cells. Their study conditions were markedly different from ours. They oversupplemented with 100 µM of FA (which is 100 times the highest FA concentration in our study (1000 nM), established ‘sufficient’ conditions as 250 nM, and intermediate conditions as 9 µM (9 times our highest concentration) of FA. Furthermore, when searched in the BLAST database their primer for detecting FR mRNA did not distinguish between α or β isoforms. Unfortunately, while they analyzed mRNA expression for FR, RFC, and PFCT for HK-2, they did not analyze FR expression for Caco-2. In a folate deficiency study from our lab, FRα mRNA expression in Caco-2 cells grown in folate deficient RPMI-1640 resulted in a non-significantly lower expression level relative to cells grown in folate replete RPMI-1640 (2.3 µM FA) (29). These findings are consistent with the present findings; at the lowest concentration of FA, 5 nM, mRNA expression of FR was significantly lower than expression at 1000 nM. In regard to RFC
mRNA expression, decreased expression was seen with increasing FA supplementation. This is consistent with previous studies (29, 112, 114) and may be due to RFC’s low affinity to FA. It may be that as expression of FR increases, the need for RFC decreases. This has yet to be investigated and established in human intestines.

There was no specific trend for PCFT expression in response to increasing FA supplementation in the present study. In general, mRNA expression levels appeared to remain consistent across FA treatments. This may mean that there are post-transcriptional modifications in PCFT proteins as seen by Dev et al (23). In their rat model, acute high FA supplementation resulted in reduced PCFT and RFC protein expression while mRNA expression levels did not change. This may also mean that expression levels of PCFT, FRα, and RFC are sufficient for folate transport in the given conditions.

Comparisons between supplementation levels within the same folate form in the present study reveal a general decrease in FPGS expression with increasing supplementation. FPGS is important for cellular folate retention and has a higher affinity to folate with shorter polyglutamate tails (141, 142). The literature suggests that during low extracellular folate concentrations, folate transport is the rate limiting step in intracellular folate accumulation (141, 142). However, during high extracellular folate conditions, FPGS becomes the rate limiting step in intracellular folate accumulation (142). Furthermore, it is postulated that lower extracellular folate concentrations can increase intracellular folate retention more efficiently than high levels of extracellular folate (141,142). In relation to intracellular folate concentrations and supplemental levels, FPGS expression was highest at 5 nM FA treatments when the intracellular and extracellular folate concentrations were the lowest. Conversely, FPGS expression was the lowest at 1000 nM FA treatments when intracellular and extracellular folate concentrations were
the highest. FPGS is also modulated by intracellular folate concentrations. That is, at high levels of intracellular folate, *FPGS* is downregulated to reduce intracellular folate retention.

Overall, results from the present study suggest that there were supplemental effects of FA on folate transporter expression. *PCFT* and *RFC* mRNA expression were higher in cells treated with 5MTHF than equimolar FA. These transporters may be crucial in 5MTHF transport over FA as intracellular folate concentration data show intracellular concentrations were generally higher in cells treated with 5MTHF rather than FA.

4.5.2  *FA vs. 5MTHF: cell growth, intracellular folate, folate metabolism, DNA synthesis*

Growth curves, intracellular folate concentrations, *DHFR, SHMT, TS*

For both cell lines, 5MTHF supplementation had higher growth rates than those grown in corresponding equimolar concentrations of FA. Growth data for HCT116 cells show that increasing equimolar concentrations of both FA and 5MTHF can increase cell growth. This was only true for Caco-2 cells treated with 5MTHF. Providing folate as 5MTHF, the bioactive form, may have increased growth rates compared to FA.

Caco-2 supplemented with increasing FA may not have shown a dose response because the extracellular and intracellular folate concentrations at the highest supplemental levels of FA may not have been sufficiently high enough to accelerate cell growth. There might be a minimum intracellular concentration of bioactive folate above which cells grow in an accelerated rate above the basal rate. Intracellular bioactive folate concentrations in FA supplemented cells may not have exceeded this threshold but intracellular folate concentrations resulting from 5MTHF supplementation likely exceeded this threshold, thereby accelerating cell growth.
In regard to bioactive folate, 5MTHF is the preferential and readily available bioactive form of folate for cell growth. 5MTHF is already reduced and can be incorporated into the folate metabolic cycle immediately. FA is a synthetic version of folate and must be reduced twice to become THF, a bioactive and naturally occurring folate. It may be that increasing concentrations of the 5MTHF in cell culture media results in a larger pool of bioactive folate entering the cell. This would theoretically lead to efficient and increased cellular metabolism of folate necessary for cell growth. *L. rhamnosus*, the bacterial strain used in our intracellular folate assay (143), is able to grow in response to all monoglutamate forms of folate as well as FA, DHF, and THF. Thus, while our assay showed that intracellular folate concentrations did increase as FA supplementation increased, this does not necessarily imply that functional and bioactive folate concentrations increased at the same level. DHFR activity is extremely low in humans and its reduction activity is the rate-limiting step in folate metabolism (6). Therefore, while increasing FA supplemental concentrations increased intracellular folate concentrations, the intracellular bioactive and functional folate pool may not be significantly different among cells grown in 4 increasing concentrations of FA. To support this, *DHFR* mRNA expression levels did not change significantly regardless of supplemental levels or folate forms in the present study for Caco-2.

In regards to HCT116, mRNA expression of *DHFR, SHMT*, and *TS* do not offer much explanation for the increase in growth and intracellular folate concentrations. It is seen however that compared to 50 nM FA, 1000 nM FA had higher *TS* expression levels. This may explain why supplementing with increasing doses of FA resulted in increased growth rates. Compared to 5nM 5MTHF, 1000 nM 5MTHF resulted in higher *SHMT* expression levels. Our study is consistent with a previous finding that higher folate intakes were associated with elevated *SHMT* mRNA (137). However, the lack of clear expression patterns makes it very difficult to draw any
conclusions. The folate levels used in this study may not have been different enough to illicit a gene expression response and instead, post-translational regulation and protein activity regulation may have played a larger role in modulating folate metabolism supporting growth.

5MTHF supplementation significantly increased growth and intracellular folate concentrations compared to equimolar FA in Caco-2 and HCT116, which cells are transformed colorectal cancer cells. It is clear that 5MTHF is at least or even more effective than FA in increasing folate status and may be an effective means of treating folate deficiency. However, with the current supplemental FA intakes, it is important to consider the consequences of providing 5MTHF in equimolar doses. If 5MTHF can increase the growth rates of these cancer cells in vitro, this may also be translated in vivo.

4.5.3 FA vs. 5MTHF: DNA methylation and methionine cycle

Global DNA methylation Methionine cycle (MTR, MTRR, MTHFR), DNA methylation (MBD2, DNMT1, DNMT3a, DNMT3b)

The effect of 5MTHF compared to FA on global DNA methylation was highly pronounced. Folate form also affected mRNA expression. Supplementation with 5MTHF generally resulted in greater expression of genes involved in the methionine cycle compared to FA. However, folate form did not affect MTHFR expression in HCT116 cells. For both HCT116 and Caco-2 cell lines, patterns of expression in relation to dose were not clear. However, the general findings still provide some evidence for the potentially superior efficacy of 5MTHF supplementation in contributing to the methionine cycle and upregulating these genes.

Similar to the methionine cycle, there were clear effects of folate form on genes involved with DNA methylation for Caco-2 cells and to a lesser degree in HCT116 cells. It is clear that for
Caco-2 cells, 5MTHF supplementation, compared to FA, is highly involved with DNA methylation. For almost all equimolar concentrations of folate, 5MTHF elicited higher $DNMT1$, $DNMT3a$, and $DNMT3b$ mRNA expression in Caco-2. In HCT116, only higher concentrations of 5MTHF resulted in higher $DNMT1$, $DNMT3a$ and $DNMT3b$ compared to equimolar FA concentrations.

Indeed, these gene expression differences were reflected in global DNA methylation patterns. Caco-2 cells supplemented with 5MTHF had higher global DNA methylation compared to cell supplemented with equimolar FA by day 7 (5 nM and 1000 nM) or day 14 (50 nM and 500 nM). Interestingly, 5MTHF supplementation in HCT116 cells had the opposite effect. HCT116 cells supplemented with 5MTHF had lower global DNA methylation compared to cells supplemented with equimolar FA (5 nM and 1000 nM) at both day 7 and day 14.

These findings have great implications for the potential of 5MTHF supplementation to modulate epigenetic patterns over FA. With emerging evidence showing that high FA intakes and high folate status may affect DNA methylation and epigenetics, offspring health outcomes, and cancer outcomes, it is important to further investigate these effects when supplemented with 5MTHF.

4.6 Conclusion

It was expected that with increasing supplementation for both FA and 5MTHF, Caco-2 and HCT116 cell lines would grow faster and result in increasing intracellular folate concentrations. Overall, it was clear that increasing folate supplementation increased cellular growth and folate concentrations. However, it was not expected that 5MTHF supplementation would result in cells growing faster than cells supplemented with FA. This finding shows that
5MTHF is indeed a readily available folate form and can be an effect form of supplementation. It also informs us that more research is required in order to determine a safe dose which will not result in adverse effects such as increased cancer growth.

It was expected that folate form would not affect gene expression and that supplementation of FA and 5MTHF would significantly decrease steady-state mRNA expression levels of genes involved in folate uptake and retention. However, this was not consistently found for all transporter genes. Only RFC decreased in expression with increasing FA supplementation for both cell lines and only PCFT followed this trend in HCT116 cells. 5MTHF did not appear to affect the expression of these genes.

It was also expected that steady-state mRNA expression levels of the selected genes involved in folate absorption, uptake and intracellular folate metabolism and one-carbon transfer reactions would not be significantly different between FA and 5MTHF. However, differences were clearly observed. The effect of folate form was most clearly observed in genes involved with DNA methylation and the methionine cycle in Caco-2 cells and, to a lesser degree, HCT116 cells.

In most of the gene expression data concerning folate uptake/transport, folate retention/hydrolysis, folate metabolism, DNA synthesis, methionine cycle, and DNA methylation, no clear dose-responsive relationship was observed with either FA or 5MTHF. Yet significant differences between FA and 5MTHF were observed with respect to folate uptake/transport, folate retention/hydrolysis, folate metabolism, DNA synthesis, methionine cycle, and DNA methylation. The methionine cycle, DNA methylation was highly favoured when supplemented with 5MTHF compared to FA. This is supported my global DNA
methylation data which show that folate form has a clear effect on the degree of methylation. These findings are also applicable to intracellular folate concentrations and growth data. Finally, it is clear that increasing folate supplementation increases intracellular folate concentrations and leads to increased cellular growth.

While overall findings were similar between Caco-2 and HCT116 cell lines, not all specific findings were consistent. These may be explained by the different morphologies exhibited and different doubling times of the two cell lines. Caco-2 are larger cells which form tight junctions to mimic enterocyte morphology and is highly adherent whereas HCT116 are smaller cells and less adherent to neighbouring cells compared to Caco-2. Due to Caco-2’s larger size, its folate requirements and uses may be different from HCT116. The doubling time for Caco-2 is typically 62 hours while HCT116 has a doubling time of 21 hours. Caco-2 may be a better model for long-term folate supplementation and HCT116 may be a better model for short-term folate supplementation. Alternatively, Caco-2 may be a better cell line to address absorption and folate use while HCT116 might be a better model to address cellular folate use when the cell is heterozygous for MTHFR. Furthermore, using these two colon cancer cell lines may help to confirm if our findings are applicable in physiological settings. If similar observations are seen in both cell lines, then we can confidently hypothesize that these findings will be consistent physiologically. If findings are contradictory between the two cell lines, then it is still uncertain if the results are applicable physiologically. For example, cellular growth and intracellular folate concentrations were generally consistent between the two cell lines. We may infer that in a physiological model, this can occur consistently. However, our global DNA methylation data showed contradictory results between the Caco-2 and HCT116 cell lines. Thus, we cannot
confidently hypothesize that global DNA methylation will be affected by our treatments in an *in vivo* model.
Chapter 5  General Discussion and Future Directions

5.1  General Discussion and Summary

We attempted to set up an in vitro model representative of the Canadian population’s median plasma folate concentration and investigate how deficient (5 nM), control (50 nM) and high (500 nM and 1000 nM) doses of folate affect cellular growth, intracellular folate concentrations, mRNA expression, and global DNA methylation. The present study provides novel data concerning differences between FA and 5MTHF supplementation on these parameters. It was clear that increasing supplementation of folate results in increased cell proliferation. Unexpectedly, folate form had an immense effect on cellular growth rates. 5MTHF, as hypothesized by many, was truly effective in raising cellular folate concentrations. However, this does not necessarily mean that 5MTHF is superior. It was also found that 5MTHF supplementation could result in higher global DNA methylation compared to FA supplementation in Caco-2 cells or lower global DNA methylation in HCT116 cells. These results provide reason to closely study and monitor how these two different forms of folate supplementation should be appropriately and safely administered.

Folate deficiency has proven to be harmful to health, however, it is increasingly seen that high folate status does not equate to greater health. High folate has been associated with increased cancer progression, perturbed methylation patterns, negative consequences in offspring health, and decreased immune function (15). Currently in the post-fortification era, 30-40% of the North American population consume FA-containing supplements (7). Multivitamin supplement use is also common among cancer patients and survivors (7) and women of child-bearing age are advised to consume a daily supplement containing 400 µg of FA in addition to folate rich foods yet most prenatal multivitamin supplements in Canada contain ~1 mg of FA.
Consequently, intake and blood levels of folate and FA have significantly increased with less than 1% deficient in the Canadian population (4).

FA and UMFA has been blamed for adverse health effects and yet these effects have not been proven to be specific to FA or UMFA. It is uncertain if adverse health outcomes may be in part due to high folate intake and high folate status in general. Yet, many nutritionists advocate for the use of 5MTHF for supplementation and 5MTHF has become available in prenatal vitamins, prescribed medical foods, and in over-the-counter supplements within minimal regulation. From the results of this study, 5MTHF supplementation is at least as efficacious as FA in treating folate deficient patients but may be harmful for those with adequate folate levels or those at risk of cancer.

My study showed that supplementation with 5MTHF resulted in higher DNA methylation compared to equimolar FA supplementation in Caco-2 cells and lower DNA methylation in HCT116 cells. As discussed earlier, in vivo studies have shown that high methyl donor supplement diets of mothers during conception result in epigenetic changes in offspring. Similarly, high doses of 5MTHF supplementation in vivo may greatly effect DNA methylation regulation resulting in perturbed gene expression. In consideration of the dual modulatory role of folate and high supplement use of cancer patients and survivors, 5MTHF supplementation at doses seen with FA, may also increase the development of established (pre)neoplastic lesions to full blown cancer and may increase cancer progression rates. This research has implications on the safety and efficacy of 5MTHF and FA in public health. It is important to clearly monitor the use of 5MTHF and elucidate the benefits and risks of 5MTHF at supplemental doses.
5.1.1 Strengths and Limitations

This study is the first study to conduct a comprehensive analysis of the effects of FA vs. 5MTHF supplementation on cellular growth, DNA methylation, and gene expression of key folate metabolism enzymes. Two different cell lines, Caco-2 and HCT116, were used for consistency and comparability with previous studies conducted within this lab. They were also used for their different characteristics even though they are both colon cancer cells. Media concentrations of folate were selected in consideration of physiologically and nutritionally relevant folate concentrations in humans post FA fortification. Many previous studies compared cells grown in folate replete medium (2.3 µM to 10 µM) with cells grown in deficient medium to implement deficiency models. By acclimatizing our cells to 50 nM FA for 5 days, we attempted to mimic the median folate serum concentrations found in the Canadian population. Our qPCR data showed interesting yet non-conclusive trends regarding folate transporters, DHFR, and folate hydrolases. Studies in the literature which found clear differences in mRNA expression used more extreme differences in supplemental conditions. In the future, comparison between more extreme folate concentrations may shed more light on these relationships.

A major limitation of my study is that as an in vitro study, it is difficult to translate findings to clinical practice. It is unknown what the optimal folate conditions are in vitro. What is known is that nutrient requirements and proliferation rates of cultured cells are extremely high, making it difficult to gauge timing and dose of interventions. We attempted to use 50 nM FA as control. However, intracellular folate concentrations of cells grown in 50 nM FA decreased over 14 days. This suggests that 50 nM FA is not a suitable control concentration to use. Another limitation is that Caco-2 and HCT116 cells are transformed cell lines and can behave differently from primary non-transformed, non-immortalized cells. Thus, using primary cell culture or non-
transformed cell lines may provide a more clinically translatable understanding of folate metabolism. Finally, mRNA gene expression data was not confirmed through protein expression and activity analysis. The functional effects of my study on cellular growth, intracellular folate concentrations, and global DNA methylation may be explained further through protein expression data. Despite these limitations, my findings provide grounds for further investigation.

5.2 Future directions

There are many ways to follow-up with my findings in *in vitro* and *in vivo* studies in regards to protein expression, downstream DNA outcomes, translatability in physiological settings, and cancer treatment response. Firstly, from this *in vitro* model, confirming the functional ramifications of mRNA expression data through protein expression and activity analysis will provide a better understanding of cellular metabolic responses to folate as well as post-translational regulation. Secondly, comparing folate form on DNA methylation or TS expression for downstream effects on DNA integrity and repair will increase our understanding of the differences in how FA and 5MTHF may be used. Thirdly, while global DNA methylation was affected by folate form, understanding if folate form modulates gene specific methylation and epigenetic patterns will be key in determining the efficacy and safety of both folate forms. Finally, an assessment of how the folate concentrations compare in the media when it is added to the cells and when the media is changed would provide some insight on how much folate is being used, oxidized, and absorbed by the cells. This is particularly important for 5MTHF in the media as it is less stable than FA in solution.

It would be interesting to see how FA and 5MTHF supplementation compares in an animal model using the same outcome measures from this study. Even further, it would be interesting to see how cancer progression is modulated by folate form. This can be studied by inducing polyps
or graft cancer cell lines into mice and see how diet supplementation affects the growth of these
tumours. Finally, it would be interesting to see how supplementation of these folate forms may
affect chemosensitivity of cancer cells. If it is true that 5MTHF supplementation results in an
increase in cellular growth rates and perturbed DNA methylation regulation compared to
equimolar doses of FA, it is vital to place regulations and recommendations to guide appropriate
administration of this folate form.
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