THE EFFECT OF FOLIC ACID SUPPLMENTATION ON CHEMOSENSITIVITY TO 5-FLUOROURACIL IN A XENOGRAFT MODEL OF HUMAN COLON CARCINOMA

by

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ABSTRACT

Folate blood levels in North America have dramatically increased over the past decade owing to folic acid (FA) fortification and widespread supplement use. Furthermore, over 50% of newly diagnosed colorectal cancer (CRC) patients use vitamin supplements containing FA while receiving chemotherapy whose mechanisms of action are based on interruption of folate metabolism. This study therefore investigated whether FA supplementation can affect chemosensitivity of human colon cancer cells to 5FU, the cornerstone of CRC treatment, using a xenograft model. FA supplementation was associated with a non-dose dependent decrease in chemosensitivity, where mice receiving 8 mg FA did not respond to 5FU and had greater tumor growth with treatment, compared to 2 (control) or 25 mg FA. Results of this study pose concern given the drastically increased intake of FA, particularly among recently diagnosed CRC patients, and from mandatory fortification. Further studies are warranted to confirm our findings and to elucidate underlying mechanisms.

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5FU	5-fluorouracil
ABC	ATP-binding cassette transporter
ACF	Aberrant crypt foci
AICART	Aminoimidazolecarboxamide ribonucleotide formyltransferase
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BAX	Bcl-2-associated X protein
BCRP	Breast cancer resistance protein
BDR	Basal dietary requirement
CHD	Coronary heart disease
CIMP	CpG-island methylator phenotype
CIN	Chromosomal instability
CpG	Cytosine-phosphodiester-guanine
CRC	Colorectal cancer
Ct	Threshold cycle
CVD	Cardiovascular disease
d(G,C,A)TP	Deoxy(guanine, cytosine, adenine) triphosphate
DCC	Deleted in colon cancer
DFE	Dietary folate equivalents
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DMG	Dimethylglycine
DMH	1,2-dimethylhydrazine
DNMT	DNA methyltransferase
DPD	Dihydropyrimidine dehydrogenase
DRI	Dietary reference intake
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
DV	Daily value
EGFR	Epithelial growth factor receptor
FA	Folic acid
FAP	Familial adenomatous polyposis
FdUMP	Fluoro-deoxyuridine monophosphate
FdUTP	Fluoro-deoxyuridine triphosphate
FOLFIRI	Folinic acid-5FU-irinotecan
FOLFOX	Folinic acid-5FU-oxaliplatin
FPGS	Folylpolyglutamate synthetase
FR	Folate receptor
FUTP	Fluorouridine triphosphate
GARFT	Glycinamide ribonucleotide formyltransferase
GGH	Gamma-glutamyl hydrolase

HLTF	Helicase-like transcription factor
HNPCC	Hereditary nonpolyposis CRC
HPF	High power field
IGFII-R	Insulin growth factor 2 receptor
IP	Intraperitoneal
JPS	Juvenile Polyposis Syndrome
KRAS	Kirsten rat sarcoma viral oncogene
LOH	Loss of heterozygosity
LV	Leucovorin (5-formylTHF)
MAP	MYH-associated polyposis
MBD2	DNA demethylase
MDR	Multidrug resistance
MGMT	O-6-methylguanine-DNA methyltransferase
MMR	Mismatch repair
MRP	Multidrug resistance protein
MS	Methionine synthase
MSI	Microsatellite instability
MTHFR	Methyltetrahydrofolate reductase
MTX	Methotrexate
NTD	Neural tube defect
OPRT	Orotate phosphoribosyl transferase
PABA	Para-aminobenzoic acid
PCFT	Proton-coupled folate receptor
PJS	Peutz-Jegher syndrome
RA	Rheumatoid arthritis
RDA	Recommended daily allowance
RFC	Reduce folate carrier
RTV	Relative tumor volume
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SHMT	Serine hydroxymethyltransferase
SNP	Single nucleotide polymorphism
TGFβII-R	Transcription growth factor beta 2 receptor
THF	Tetrahydrofolate
TK	Thymidine kinase
TNM	Tumor node metastasis (staging system)
TP	Thymidine phosphorylase
TS	Thymidylate synthase
UK	Uridine kinase
UMFA	Unmetabolized folic acid
UMPK	Uridine monophosphate kinase
UP	Uridine phosphorylase
VEGF	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

Folate is a water soluble B-vitamin that is found naturally occurring in green leafy vegetables, citrus fruits, legumes and organ meats. Folic acid (FA), the synthetic and more bioavailable form, is found in fortified foods and dietary supplements. As an important mediator of one-carbon transfer reactions involved in nucleotide synthesis and biological methylation reactions, folate plays a vital role in cell division, DNA repair, and epigenetic control [1]. As a result of FA fortification of the food supply, the prevalence of neural tube defects (NTDs) has decreased by nearly 50% in Canada [2]. This public health initiative was intended to provide an additional 100-200 µg per day, but population data suggest that total FA intake has increased by greater than 300 µg dietary folate equivalents (DFE). Erythrocyte folate concentrations accurately reflect increases in intake and show a 50% increase in concentrations [3].

In addition to FA fortification, approximately 50% of the Canadian and United States population regularly use dietary supplements [4, 5], and is the primary predictor of total FA intake [6, 7]. Dietary supplements or multivitamins contain a minimum of 400 µg, and up to 1 mg FA, which is the upper tolerable limit, set by the Institute of Medicine [8]. Among cancer patients and survivors, dietary supplement use is prevalent in up to 81% of the population [9]. Studies suggest that up to 68% of physicians are unaware of their patients' supplement use, and it is often an autonomous decision by the patient [10]. Recently diagnosed cancer patients are particularly susceptible to advertisements or other promotional articles claiming health promotion activities, often which have little to zero scientific evidence supporting its possible anticancer effects [11]. In fact, folate deficiency is virtually nonexistent in Canada and 40% exhibit levels above 1360 nmol/L, the cut-off level for high blood folate [12].

FA supplementation have shown to be effective in correcting megaloblastic anemia and preventing neural tube defects (NTDs) [1]. However, there is a growing body of evidence that FA at high doses can cause adverse affects on human health, such as the promotion of existing cancers [13]. The relationship between folate and colorectal cancer (CRC) is the most studied. A large body of epidemiological evidence suggests that a modest dose of FA supplementation can reduce the risk of CRC by 20-40% [13, 14]. However, preclinical animal studies demonstrate that the relationship is complex and in fact, folate plays a dual modulatory role depending on the dose and stage of transformation at the time of folate intervention [13]. In normal cells, folate deficiency predisposes cells to neoplastic transformation, whereas modest levels of FA supplementation may prevent neoplastic transformation [13, 15]. Once (pre)neoplastic cells are established, however, folate deficiency suppresses, whereas FA supplementation promotes, the progression of (pre)neoplastic lesions [13, 16]. Inducing a state of folate deficiency effectively limits substrates for nucleotide synthesis, inhibiting tumor growth. This has been the molecular basis for the use of antifolate chemotherapy.

5-fluorouracil (5FU) is an antimetabolite of folate metabolism. Its main anticancer effect is induced through the formation of a stable ternary complex with thymidylate synthase (TS) to inhibit thymidine synthesis [17]. Adequate concentrations of 5,10-methyleneTHF play a critical role in the stability of the 5FU-TS complex [18] and as a result, 5-formylTHF, or leucovorin (LV) is administered one hour prior to 5FU to selectively expand the intracellular pools of 5,10-methyleneTHF. Given that the efficacy of 5FU depends on intracellular folate concentrations, it is a biologically plausible mechanism that high levels of FA supplementation can interfere with chemotherapeutic agents that are based on the interruption of folate metabolism. Recent *in vitro*

evidence suggest that there are mechanisms of drug resistance that can be induced by elevated concentrations of intracellular folate [19].

To our knowledge, no studies have been conducted to evaluate the effects of FA supplementation at levels equivalent to those achieved in the postfortification era on chemosensitivity to 5FU-based treatments. Thus, the main objective of this thesis was to determine whether FA supplementation in a xenograft model of human colon carcinoma could decrease chemosensitivity to 5FU. An additional objective was to identify potential mechanistic pathways of multidrug resistance (MDR) which could be induced by FA supplementation and confer resistance to chemotherapy.

CHAPTER 2: LITERATURE REVIEW

2.1. Colorectal cancer

2.1.1. An overview of colorectal cancer

Colorectal cancer (CRC) ranks third in incidence in Canada, trailing prostate and lung cancer in males, and breast and lung cancer in females [20]. The Canadian Cancer Society estimates 23 300 Canadians will be diagnosed, and 9 200 will die in 2012 [20]. That is, one in thirteen males and one in sixteen females are expected to develop CRC during their lifetime [20]. Though international incidence greatly varies by age, sex, and ethnicity, mortality has decreased to 33.4% and 28.4% for men and women, respectively [21]. Despite the decreasing rates, CRC contributes a significant economic impact on the healthcare system, estimated to cost $14.03 billion by the year 2020 [22].

The etiology of CRC involves a complex interaction between genetics and environmental factors but can be classified into familial or sporadic. CRC syndromes recognized as heritable include familial adenomatous polyposis (FAP), MYH-associated polyposis (MAP), hereditary nonpolyposis CRC (HNPCC), Peutz-Jegher syndrome (PJS), and juvenile polyposis syndrome (JPS) (**Table 2.1**). Individuals with a family history of these syndromes are at an increased risk of inheriting germline mutations in genes such as *APC*, *MYH*, and those involved in mismatch repair (MMR) and overall maintenance of DNA integrity. Approximately 25% of CRC patients have a family history of CRC suggesting that genetics play an important role in incidence. An immediate family member afflicted with CRC increases an individual's risk by threefold [23]. The remaining 75% of CRC cases are considered sporadic, which are seemingly spontaneous in nature, but are thought to arise from an interaction between age, genetics, and environmental factors.

Table 2.1. Hereditary colorectal cancer syndromes and characteristics [24]**.**

Syndrome	Frequency Lifetime risk	Heritability	Genetic characteristics	Functional consequence
Familial adenomatous polyposis (FAP)	1% 85-100% risk	Autosomal dominant	Mutation in *APC*	Multiple polyps in colon
MYH-associated polyposis (MAP)	1% 85-100% risk	Autosomal recessive	Mutation in *MYH*	Defective base-excision repair Multiple polyps in colon
Lynch syndrome (HNPCC)	1-5% Up to 80% risk	Autosomal dominant	Mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2*	Defective MMR machinery
Peutz-Jegher syndrome	0.004-0.003% Up to 95% risk	Autosomal dominant	Mutation in *STK11*	Hamartomatous polyps in GI tract
Juvenile polyposis syndrome	0.02-0.01% 9-50% risk	Autosomal dominant	Mutation in *BMPR1A*, *SMAD4*	Hamartomatous polyps in GI tract

2.1.2. Colorectal cancer carcinogenesis

The earliest known precursors of CRC are aberrant crypt foci (ACF). These lesions are aggregates of abnormal crypts which can appear elevated, depressed or flat in phenotype. ACFs are apoptosis-resistant, tend to have larger crypts, nuclei stratification and an elongated, almost elliptical, luminal opening [25]. A wide range of histopathological characteristics, ranging from hyperplasia to dysplasia, have shown varying degrees of potential for malignancy. Dysplastic ACFs with a hyperproliferative surrounding epithelium, usually located in the distal colon, are generally accepted as precursors of adenomatous polyps and subsequently adenocarcinomas [25]. Adenomatous polyps or adenomas are protrusions of proliferative epithelial tissue which have a high likelihood of progressing to cancer and are removed during colonoscopies. Incidences of adenomas are high among those who have previously had adenomas, CRC, and those over fifty years of age [23]. Hyperplastic or inflammatory polyps are benign and generally do not confer an increased risk for CRC. Adenomas develop slowly over five to ten years, and continue to grow and acquire molecular and dysplastic changes and become malignant, if not detected and

removed. Once malignant, adenocarcinomas invade mucosa, submucosa, muscularis propia, and serosa, and metastasize to local lymph nodes and subsequently to distant organs. The age of the individual and stage of diagnosis have a significant impact on five-year survival. Cases are diagnosed based on the Tumor Node Metastasis (TNM) staging system which considers tumor invasion, lymph node involvement, and the presence or absence of metastasis. TNM stage I tumors are limited to the mucosa and submucosa, without lymph node involvement or metastasis, and have a five-year survival rate greater than 90% [20]. TNM stage II tumors have invaded to the muscularis propia or serosa, however still lack lymph node involvement or metastasis, and the five-year survival rate ranges from 55-85% [20]. TNM stage III tumors exhibit tumor invasion and lymph node involvement, but no metastasis, and thus the five-year survival rate decreases to 20-55% [20]. Finally, TNM stage IV tumors exhibit tumor invasion, lymph node involvement and metastasis to distant organs. Cases as such, have less than a 5% five-year survival rate [20]. In addition, the location of adenocarcinomas greatly impacts survival rates, where CRC in the proximal colon have a better prognosis than those found in the distal colon [26]. The first and foremost treatment option is surgical resection. Adjuvant chemotherapy is considered for individuals with stage III and IV CRC, and selected patients in stage II (**Table 2.2**).

Table 2.2. Approved treatment regimens for colorectal cancer [27].

Treatment	Description	**Stage** Five-year survival rate			
		I > 90%	**II** 55-85%	**III** 20-55%	**IV** < 5%
Surgical resection	Removal of cancerous lesions, followed by anastomosis	X	X	X	X
Fluorouracil	IP infusion folinic acid (LV), followed by IP bolus 5FU		X	X	X
FOLFOX4	FOLinic acid-Fluorouracil-OXaliplatin (low dose)			X	X
FOLFOX6	FOLinic acid-Fluorouracil-OXaliplatin (high dose)			X	X
FOLFIRI	FOLinic acid-Fluorouracil-IRInotecan			X	X
Capecitabine or 5FU	Capsule, oral dosing			X	X
Irinotecan	IP infusion irinotecan			X	X
CAPOX	CAPecitabine-OXaliplatin				X

2.1.3. Risk factors of colorectal cancer

Several risk factors have been identified to play a role in colorectal carcinogenesis. Non-modifiable risk factors include genetic predisposition (CRC syndromes and family history of CRC or other cancers), age, previous history of adenomas or CRC, and chronic inflammatory bowel disease [21, 23]. Modifiable risk factors include physical inactivity, frequent alcohol consumption, tobacco use, and excessive weight [21, 23]. In addition, a diet low in fibre, a low consumption of fruits and vegetables and a high intake of red meat appear to contribute to the development of CRC [21, 23]. Lifestyle habits commonly associated with the Western world (high animal and saturated fat, high simple sugar, low fibre, and a sedentary lifestyle) that are associated with insulin resistance, are generally considered a unifying pathogenetic factor in the development of CRC [23].

2.1.4. Molecular events of colorectal cancer

The transformation of normal epithelial cells to malignant cells is governed by a series of molecular genetic and epigenetic events leading to histopathological changes. In general, the adenoma-carcinoma sequence follow one of two molecular pathways, leading to phenotypes exhibiting microsatellite instability (MSI), chromosomal instability (CIN), or CpG island methylator phenotype (CIMP) (**Figure 2.1**).

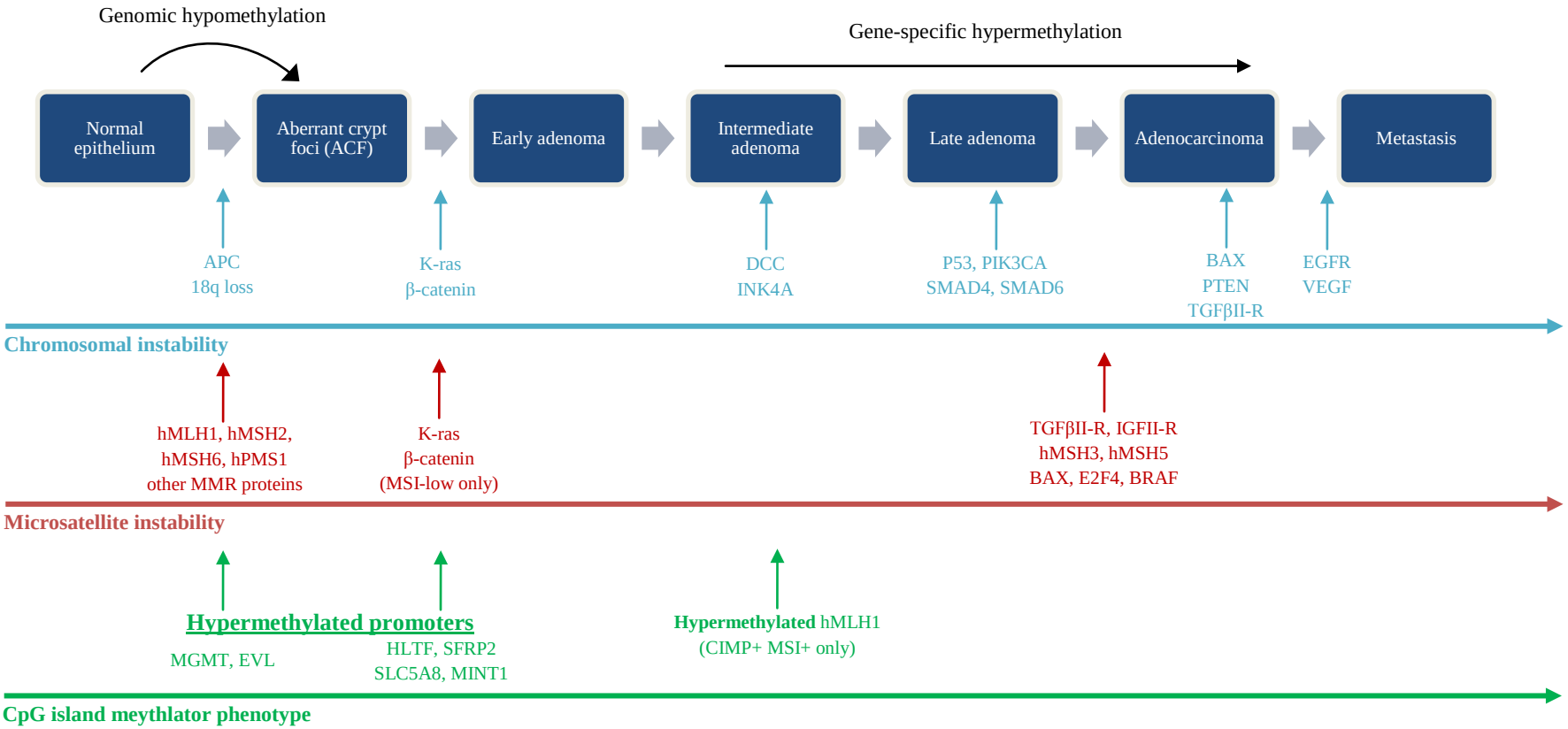

Figure 2.1. Molecular events of colorectal carcinogenesis, classified by phenotype. Chromosomal instability (CIN; blue) is identified by the loss of heterozygosity (LOH) of *APC* and/or loss of chromosome 18q, causing karyotypic abnormalities and instability [28]. Loss of APC function onsets hyper-activation of the Wnt signaling pathway (K-ras, β-catenin). As the cancer progresses, mutations in genes responsible for tumor suppression (*DCC*, *INK4*, *TP53*), cell growth, adhesion and proliferation (*PIK3CA*, *EGFR*, *VEGF*), apoptosis (*BAX*, *PTEN*) and general transcription factors (*SMADs*, *TGFII-R*) occur, promoting oncogenesis. Microsatellite instability (MSI; red) is identified by the hereditary mutation in mismatch repair (MMR) proteins (h*MLH1*, h*MSH2*, h*MSH6*, h*PMS1*) [29]. Activation of the Wnt signaling pathway is seen in MSI-low, but not MSI-high phenotypes [30, 31]. Defective MMR machinery cause

aberrant lengthening and shortening of microsatellites compromising genomic stability and further mutations in genes involved in DNA repair (h*MSH3* and h*MSH5*), growth factors (*TGFβII-R*, *IGFII-R*, *E2F4*), apoptotic proteins (*BAX*), and cell signaling and growth (*BRAF*). Ineffective BRAF is a common characteristic among spontaneous MSI cases. CpG island methylator phenotype (CIMP; green) is characterized by distinct patterns of hypermethylation at CpG islands located in promoter regions [32]. The trigger of CIMP onset is largely unknown, but hypermethylated promoters of *MGMT* and *EVL* are considered a prognostic marker of this phenotype [33]. MGMT is responsible for the removal of O^6-alkyl-guanine, a DNA adduct, and EVL is involved in maintenance of the extracellular matrix. Subsequent aberrant promoter methylation of *HLTF*, *SFRP2*, *SLC5A8* and *MINT1* are likely to contribute to suppression of tumor suppressor genes [32, 34]. Modified from [28, 29, 35].

2.1.4.1. Microsatellite instability

MSI is characterized by mutations in the MMR genes and aberrant lengthening and shortening of microsatellites, located within the DNA [29]. These regions are particularly prone to mutations because the repetitive sequences make binding of DNA polymerases difficult and inefficient. Incorrectly replicated DNA often escape repair mechanisms and proceed to generate degenerate, or truncated, proteins. MMR proteins such as, MLH1, MSH2, MSH3, MSH6 and PMS2 bind to DNA in heterodimers to recognize, locate and recruit exonucleases to excise mismatched bases.

In most cases, MSI is triggered by hypermethylation of h*MLH1*, effectively preventing its transcription, and subsequent protein formation [36]. Interestingly, there have been reports of females more likely to have h*MLH1* hypermethylation compared with males [37, 38]. In other cases, there can be germline mutations in *MSH2*, which inactivate both MSH2 and MSH6, and other MMR proteins. Germline mutations can impact an individuals' risk of developing HNPCC syndrome by up to 70% [39]. Ineffective BRAF, a protein involved in cell signaling and growth, is a common characteristic among spontaneous MSI cases caused by h*MLH1* hypermethylation [40]. MSI further causes mutations in genes involved in DNA repair, growth factors, apoptotic proteins and histone modifiers [41]. Tumors of MSI origin tend to be located in the proximal colon, commonly diagnosed among stage II patients, and generally have a good prognosis with a lower probability than MSI-negative tumors to metastasize to lymph nodes and other organs [42-44]. CRCs exhibiting MSI are found in approximately 15% of all cases and have clinically been less responsive to conventional 5FU-based treatment, compared to CRCs with CIN [45]. A number of retrospective and randomized clinical trials indicate that HNPCC patients receiving irinotecan with standard treatment benefit greater than those receiving standard treatment alone

[46-48]. Irinotecan (CPT-11) inhibits topoisomerase I, which binds to DNA, nicks one strand to relieve helical tension, and aids in reannealing. CPT-11 binds to the DNA-topoisomerase I complex preventing reannealing, causing a double stranded break, which triggers cell death [46]. As well, individuals expressing the single nucleotide polymorphism (SNP) of *MTHFR* (ex. MTHFR C677T) are at a higher risk of developing MSI CRC [49]. It appears that aberrant methylation patterns of MSI become favoured in an environment where methionine is not readily regenerated from homocysteine, since altered DNA methylation is caused by disruption of folate and methyl group metabolism [49]. The distinct histopathology and phenotype of MSI hold value in its prognosis, and harbor potential for tailored therapies.

2.1.4.2. Chromosomal instability

As proposed by Fearon and Vogelstein in 1990 [50], carcinogenesis is the result of several critical gene alterations which in turn, affect a number of downstream pathways. Though it is unknown whether CIN is first triggered by karyotypic abnormalities during sister chromatid separation or mutations in key genes, it is understood that one exacerbates the other and the degree of CIN increases as carcinogenesis progresses [28]. The earliest genomic event that occurs is the loss of heterozygosity (LOH) of the *APC* gene, resulting in disruption of the Wnt signaling cascade. Products of the *APC* gene have roles in tumor suppression, cellular differentiation and adhesion, and apoptosis. Ineffective *APC* leads to an accumulation of nuclear β-catenin, normally ubiquitinated for degradation, driving the transcription of oncogenic genes [51]. These few, but critical, genomic events trigger a cellular environment increasingly vulnerable to CIN. Mutations in *KRAS* are seen in the majority of CIN cases, which activate components of the MAP/ERK pathway, implicated in tumorigenesis. Cell signal transduction by critical players in this pathway promote cell proliferation. Along the way, mutations in

transcription factors occur to ensure survival and proliferation of cancer cells, such as *EGFR*, *VEGF*, and *TGFβ* [52]. Subsequent histological changes are driven by mutations in tumor suppressor genes (*DCC*, *INK4A*, *TP53*, *SMADs*, *DPC4*) and apoptotic genes (*BAX*, *PTEN*) [52]. The CIN phenotype represents the majority of CRC cases (75-85%). The intratumoral heterogeneity proves difficult to target specific components, and as such, CIN persistently show poorer prognosis compared to MSI. A recent meta-analysis of 63 studies shows that CIN-positive tumors had significantly poor progression-free survival and an overall hazard ratio of 1.45, compared to CIN-negative tumors ($p<0.001$, 95% CI: 1.35 to 1.55) [53]. A number of studies have sought prognostic markers such as *KRAS*, p53 or 18q alterations [54-58], however it appears that it is the overall phenotype of CIN that best indicates prognosis, rather than a single mutation to target [59].

2.1.4.3. CpG island methylator phenotype

A third, and the least common, phenotype called CIMP is characterized by distinct patterns of hypermethylation at CpG islands, which are high CG density sequences primarily located upstream near promoters, exon 1 or 5' untranslated regions [60]. Hypermethylation of *IGF2*, *HIC-1*, *p16*, *THBS1*, and *COX2* are characteristic of CIMP [32, 61, 62]. In general, CIMP tumors are poorly differentiated tumors found in the proximal colon [63, 64] and exhibits wildtype *TP53*, but mutated *KRAS* [61]. The prognostic evidence of CIMP-positive tumors in response to 5FU has been conflicting since there are combinations of methylated CpG islands that may have a stronger predictive value and can vary with different panels of hypermethylated promoters. It appears that the CIMP-positive tumors could be a surrogate marker for widespread aberrations in methyl group metabolism, and such changes may render cells more sensitive to therapies based on the interruption of folate metabolism, such as 5FU [65]. Van Rijnsoever *et al*

puts forth that the increased chemosensitivity could be due to the transcriptional silencing that inactivate genes required for cell survival in the presence of 5FU [65]. More recent studies suggest that chemosensitivity of CIMP-positive tumors are dependent on mutated *BRAF*, a component of the MAP/ERK pathway, which regulates cell division, differentiation and secretion [66, 67]. A proportion of CIMP-positive tumors exhibit hypermethylation of h*MLH1*, similar to MSI, and can create difficulties in differentiating between phenotypes [36, 68].

A comprehensive examination of the phenotypes elucidate characteristic molecular genomic and epigenetic events, however similarities between MSI, CIN and CIMP result in combination phenotypes, constantly challenging proposed treatments for specific phenotypes. Identifying the phenotypes of CRC cases is an important factor in prognosis and should be taken into consideration when treatment options are discussed.

2.1.5. 5-fluorouracil based chemotherapy

5FU is the cornerstone of many chemotherapeutic regimens for CRC and are used in conjunction with other targeting agents in stages III and IV.

2.1.5.1. Mechanisms of 5-fluorouracil

5FU is a uracil analogue which is rapidly transported into the cell and converted into one of three active metabolites – fluoro-deoxyuridine monophosphate (FdUMP), fluoro-deoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The main anticancer effect of 5FU is its inhibition of thymidylate synthase (TS), a critical enzyme involved in thymidylate synthesis, via its main metabolite FdUMP [17, 69] (**Figure 2.2**). TS exists as a homodimer and under normal conditions binds 5,10-methyleneTHF and deoxyuridine monophosphate (dUMP) in equal ratios. FdUMP competitively binds to dUMP sites forming an inhibitory ternary complex, preventing deoxythymidine monophosphate (dTTP) formation for thymidylate synthesis. TS

inhibition further causes an imbalance with dATP, dGTP and dCTP, and ultimately inhibition of DNA replication. Intravenous 5FU is administered with leucovorin (LV, 5-formylTHF), a precursor of 5,10-methyleneTHF, which directly contributes to the intracellular pool of 5,10-methyleneTHF and enhances the formation and stabilizes the inhibition of the TS ternary complex [18, 70, 71]. Other mechanisms of 5FU are the misincorporation of FdUTP and FUTP in DNA and RNA, respectively [17]. These two processes cause significant disruptions in nucleotide processing and function, and the persistent damage initiates p53-mediated apoptosis [72]. The rate-limiting step in 5FU metabolism is the hepatic metabolism by dihydropyrimidine dehydrogenase (DPD), responsible for 80% of 5FU catabolism and its short half life [17]. Thus, DPD-inhibitors and other modulators of 5FU have been utilized in conjunction with treatment to prolong treatment effects without additional dosing [73].

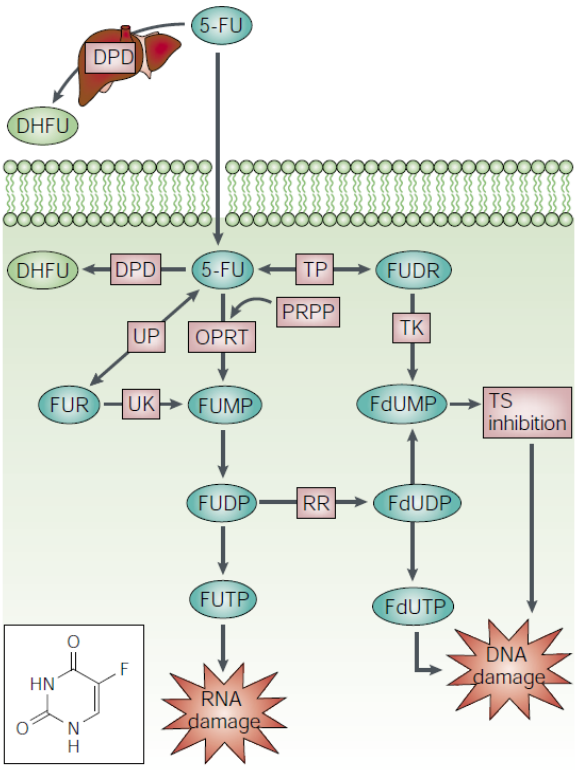

Figure 2.2. Metabolism of 5-fluorouracil. 5FU requires the catalytic conversion by uridine phosphorylase (UP), uridine kinase (UK), orotate phosophoribosyltransferase (OPRT), thymidine phosphorylase (TP), and thymidine kinase (TK) into one of its three active metabolites: FdUMP, FdUTP and FUTP. The primary mechanism is the formation of an inhibitory ternary complex by FdUMP competitively binding to TS, preventing the *de novo* synthesis of pyrimidines. Secondary mechanisms include the incorporation of FdUTP and FUTP into DNA and RNA, respectively, triggering cell death. Up to 80% of 5FU is catalyzed by hepatic dihydropyrimidine dehydrogenase (DPD). Adapted and reprinted by permission from the publisher (Nature Publishing Group): [17].

2.1.5.2. Adjuvant chemotherapy for CRC

5FU is the main component of all chemotherapy regimens used for CRC (**Table 2.2**). Capecitabine is an oral pro-drug of 5FU and has shown equal or enhanced efficacy to 5FU [74]. The addition of platinum-based drugs like oxaliplatin enhance efficacy by increasing cytotoxicity in cancer cells [75] and this combination is referred to as FOLFOX. FOLFIRI uses irinotecan instead, effectively preventing DNA from unwinding for replication. Regimens for stage IV CRC can also include monoclonal antibodies designed to bind to epidermal growth factor receptor

(EGFR) or vascular endothelial growth factor receptor (VEGFR), which are antigens often overexpressed in cancer cells [76]. EGFR is a cell surface receptor which activates upon ligand binding (EGF or TGFα) and dimerization, initiating signal transduction cascades of pathways responsible for cell proliferation, adhesion and migration. VEGF functions as an angeogenic switch, whereby binding to VEGFRs on cell surfaces elicits downstream cascades, resulting in migration and mitosis of endothelial cells, increasing endothelial permeability, and stimulating angiogenesis.

2.1.6. Mechanisms of acquired drug resistance

Drug resistance in cancer can be classified as one of two general types: genetic alterations within the cancer cell itself or, impaired delivery or sequestration of drugs within the cell. The nature of drug resistance is multifactorial and can involve blocked influx of drug, increased metabolism of drug, altered drug targets, mutations in apoptotic pathways, altered cell-cycle checkpoints, and efflux of drug [77, 78]. Specifically for 5FU, some important determinants of efficacy are activity levels of TS, TP, DPD, and p53, and the CRC phenotype. Repeated cycles of 5FU can confer resistance at the levels of TS by increasing the number of TS protein for FdUMP to bind to [79]. Omura *et al* reports that the number of FdUMP binding sites significantly increased following 5FU, suggesting that treatment can become increasingly ineffective if doses remain constant and the sites available for FdUMP or dUMP increases [79]. Furthermore, polymorphisms of the *TS* promoter have a profound effect on 5FU response, where the 3R/3R genotypes have approximately three times the TS mRNA than the 2R/2R and 2R/3R genotypes and therefore decrease chemosensitivity to 5FU [80].

TP is responsible for the reversible conversion of 5FU to fluorodeoxyuridine (FUDR), which can then be converted to FdUMP [17]. The evidence defining the role of TP in 5FU

response has been conflicting between preclinical and clinical studies. *In vitro* and *in vivo* animal studies show TP overexpression is associated with an increased sensitivity to 5FU, presumably due to increased inhibition of the ternary complex at higher FdUMP concentrations [81]. By contrast, Metzger *et al* demonstrated in a retrospective analysis of 38 tumor samples that higher TP expression is associated with reduced response to 5FU [82]. Authors suggest that this contradictory finding may be explained by the dual role of TP as an endothelial cell growth factor, which play a role in promoting angiogenesis [82].

DPD is a hepatic enzyme responsible for the catabolism of 5FU metabolites and thus individuals who are DPD-deficient, due to mutations in the DPD gene, experience systemic toxicity [83]. Intuitively, colorectal tumors expressing excessively high DPD levels are resistant to 5FU [84]. P53 is a key tumor suppressor protein and induces temporary cell cycle arrest permitting time for repair of DNA damage, and further imposes a permanent block on future cell division [85]. Mutated p53 protein leads to a disruption in these crucial regulatory processes, and tumors with abnormal p53 function result in poorer outcomes compared to tumors with normal p53 function. An extensive review on p53 abnormalities and CRC prognosis suggests there is no effect of p53 status on response to chemotherapy, however authors put forth there is substantial heterogeneity between studies as well as publication bias [55]. Despite the large number of studies that have been conducted, there is no clear evidence whether chemosensitivity to 5FU is affected by p53 status [55, 86].

ATP-binding cassette (ABC) transporters have a crucial role in the development of multidrug resistance (MDR) by controlling drug efflux (**Table 2.3**). Forty-nine members are classified into seven subfamilies (A-G) according to genetic homology. Transporters facilitate mobilization of metabolic products, lipids and sterols, proteins and amino acids, ions, metals,

saccharides and drugs [78]. These proteins are located on the nuclear or cytoplasmic membrane and harness the energy from ATP hydrolysis to remove drugs from cellular compartments or the entire cell itself [87] (**Figure 2.3**). Specifically implicated in response to 5FU chemotherapy includes ABCB5, MRP5 (ABCC5) and MRP8 (ABCC11), which selectively bind nucleotide analogs such as FdUMP. Preliminary studies show that activity, not expression, of these MDR proteins is increased following repeated cycles of 5FU. Wilson *et al* reports that tumor biopsies from CRC patients exhibit activity levels significantly increased following 5FU treatment and ABCB5-knockdown treatment significantly reduced xenograft growth and mediated cell death in response to 5FU [88]. MRP 5 and 8 have been implicated to induce resistance to 5FU by increasing efflux activity [89-92]. Pratt *et al* show MRP5 mediates the transport of FdUMP, FUMP and dUMP, but not FUDR or unmetabolized 5FU in human embryonic kidney (HEK) cells [90]. Furthermore in a three-day cytotoxity assay, MRP5-transfected cells were approximately nine-fold resistant to 5FU treatment compared to wildtype HEK cells [90]. Similarly, resistance has been shown to be conferred by MRP8 manipulation *in vitro*. MRP8-transfected kidney cells exhibited enhanced cellular extrusion of cyclic nucleotides and conferred resistance to FdUMP and FUDR by three- and five-fold, respectively [89]. Furthermore, Oguri *et al* have shown a 25-fold increase in resistance to 5FU by active efflux of FdUMP by MRP8 in a 5FU-resistant cell line [92]. Since 5FU is ubiquitously used in the treatment of CRC, identifying physiologic functions and substrates of drug efflux pumps will elucidate mechanisms of acquired drug resistance and provide possible predictive markers of chemotherapeutic efficacy.

The identification and classification of CRC based on genetic and sporadic cases have undoubtedly added to the body of knowledge in understanding important pathogenetic events of MSI, CIN and CIMP. Though molecular events and genetic alterations in the three phenotypes

can differ in many ways, 5FU remains the cornerstone of all CRC chemotherapy. Regimens can be tailored to patients with the addition of oxaliplatin, irinotecan, and monoclonal antibodies, depending on the stage of diagnosis. However, we are far from utilizing predictive markers of 5FU response and MDR to further enhance treatment. CRC treatment is not only a burden on the patient, due to toxicity-related side effects and fatigue, but adds a significant economic burden on our healthcare system. Further investigations of innate and acquired mechanisms of resistance in response to 5FU treatment will elucidate next steps towards better targeted therapies.

Table 2.3. List of selected ABC transporter proteins implicated in multidrug resistance. Adapted and reprinted by permission from the publisher (Oxford University Press): [78].

Transporter	Tissue	Non-chemotherapy substrates	Chemotherapy substrates	Reference
P-glycoprotein/ MDR1 (ABCB1)	Intestine, liver, kidney, testes, placenta, brain	Neutral and cationic organic compounds	Doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide	[93]
MDR2 (ABCB4)	Liver	Choline analogs, some hydrophobic drugs	Paclitaxel, vinblastine	[78]
ABC19 (ABCB5)	Intestine, liver	Nucleotide analogues	Doxorubicin, 5FU	[88]
MRP1 (ABCC1)	All tissues	Glutathione, organic anions	Doxorubicin, MTX	[93-97]
MRP2/cMOAT (ABCC2)	Liver, kidney, intestines	Glutathione, organic anions	Doxorubicin, MTX, cisplatin	[93, 94, 96, 97]
MRP3 (ABCC3)	Pancreas, kidney, intestine liver, adrenal glands	Glutathione, bile acids, organic anions	Etoposide, MTX, cisplatin, doxorubicin	[96, 98-100]
MRP4 (ABCC4)	Prostate, testes, ovary, intestine, pancreas, lung	Nucleotide analogues, organic anions	MTX, thiopurines	[101]
MRP5 (ABCC5)	Most tissues	Nucleotide analogues, organic anions	6-MP, 5FU	[90]
MRP6 (ABCC6)	Liver, kidney	Anionic cyclic peptides		[102]
MRP7 (ABCC10)	Most tissues	Lipophilic anions, glucuronides	Docetaxel, vinorelbine	[103]
MRP8 (ABCC11)	Most tissues	Nucleotide analogues, lipophilic anions	5FU	[89, 92, 103]
BCRP (ABCG2)	Placenta, intestine, breast, liver	Prazosin	Doxorubicin, daunorubicin, topotecan	[93, 102, 104]

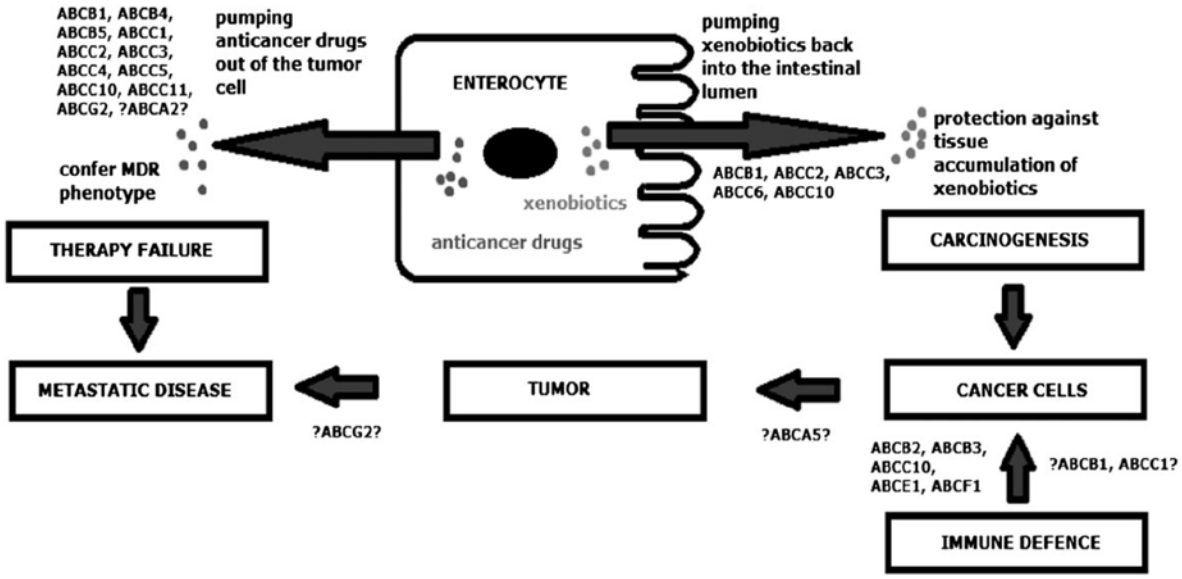

Figure 2.3. Proposed interactions and mechanisms of ABC transporter proteins in colorectal carcinogenesis. Adapted and reprinted by permission from the publisher (Oxford University Press): [105].

2.2. Folate

2.2.1. An overview of folate

Folate is a term used to describe forms of Vitamin B9 which are functionally similar and are structurally made up of a pterin ring, para-aminobenzoic acid (PABA) and up to nine glutamate residues (**Figure 2.4**). Though humans can synthesize all components, we lack the enzyme required to couple the pterin ring to PABA, thus must obtain folate from their diet [1]. Folic acid (FA) describes its fully oxidized and monoglutamated form. Naturally occurring folates (**Table 2.4**) are very unstable and rapidly lose their bioactivity. Approximately 50-75% of folate content is lost through food harvesting, storage, processing and preparation [1]. In contrast, FA remains stable for months, or even years, and has a higher bioavailability. One microgram food folate is equal to 0.6 µg FA from fortified foods or supplements taken with food, or 0.5 µg

FA of a supplement on an empty stomach [8]. Dietary reference intakes (DRI) are measured in dietary folate equivalents (DFE) to take into consideration the differences in bioavailability.

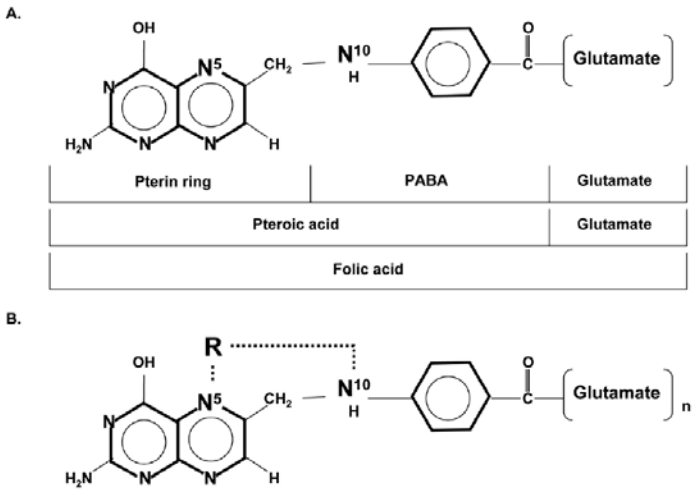

Figure 2.4. Chemical structures of folic acid (A) and folate (B). FA is made up of a pterin ring, para-aminobenzoic acid (PABA) and up to one glutamate residue. It is the fully oxidized form of folate and up to a 50% greater bioavailability that natural folates. In its reduced form, folate can have up to nine glutamate residues by gamma-peptide linkages. Side groups (R) can be added in the form of 5-methylTHF, 5,10-methyleneTHF, 5-formylTHF, and 10-formylTHF. Adapted and reprinted by permission from the publisher (John Wiley and Sons): [106].

2.2.1.1. Dietary recommendations

The recommended daily allowance (RDA) for adult men and women is 400 µg/day of DFEs. For pregnant and lactating women, the RDA increases to 600 µg and 500 µg/day, respectively, to support child growth. Health Canada recommends that all women planning a pregnancy consume a 400 µg FA supplement daily, in addition to a diet rich in food folate to support growing maternal and fetal tissue growth, and expanding maternal blood volume [107]. There is no upper tolerable limit of folate intake; however the Institute of Medicine (IOM) suggests no more than 1000 µg/day FA from fortified foods and supplements. This guideline was deemed acceptable to prevent masking of vitamin B12 deficiency [8].

Table 2.4. Selected food sources of folate and folic acid. Percent of daily value (DV) is based on a 2000 kcal/day diet. Modified from [108].

Food	Amount (µg/serving)	% DV
Excellent sources (>55 µg/serving)		
Beef liver (cooked, braised; 3 ounces)	215	54
Lentils (cooked, boiled; ½ cup)	179	45
Spinach (frozen, cooked, boiled; ½ cup)	115	29
*Breakfast cereals (fortified with 25% DV; ¾ cup)	100	25
Asparagus (boiled; 4 spears)	89	22
*Pasta, white (cooked, enriched; ½ cup)	84	21
*Rice, white (cooked, enriched; ½ cup)	77	19
Avocado (raw; ½ cup)	59	15
Spinach (raw; 1 cup)	58	15
Good sources (33-54 µg/serving)		
Broccoli (frozen, cooked; ½ cup)	51	13
Tomato juice (canned; 1 cup)	49	12
Green peas (frozen, boiled; ½ cup)	47	12
Orange juice (1 cup)	47	12
*Bread, white (1 slice)	43	11
Broccoli (raw; 2 spears)	40	10
Strawberries (raw; 1 cup)	40	10
Cantaloupe melon (raw; 1 cup)	34	9
*fortified with FA		

2.2.2. Folate metabolism

Folate absorption and bioavailability are determined by the ability to hydrolyze polyglutamate tails to three glutamate residues or less [109] (**Figure 2.5**). Natural folates must undergo hydrolysis by glutamate carboxypeptidase II (GCPII), an exopeptidase that is anchored to the intestinal apical brush border membrane of the small intestine. Mono-, di-, or tri-glutamated folates are then transported across the membrane by one of three transporters: a reduced-folate carrier (RFC), folate receptor (FR), or proton-coupled folate transporter (PCFT, SLC46A1) (**Table 2.5**). RFC is a facilitative anion exchanger, with preference for reduced folates such as 5-methylTHF, the main metabolite formed from natural folates (K_m= 1-5 µM), rather than oxidized metabolites (K_m = 100-200 µM) [110, 111].

In contrast, FR has a very high affinity for FA (K_m < 1 nM) compared to its lower affinity for 5-methylTHF (K_m = 3 nM) [110, 111]. Four isoforms of FR exist (α, β, γ, γ_1) however only the α and β isoforms are membrane bound, and only the α isoform is involved in folate uptake [110, 111].

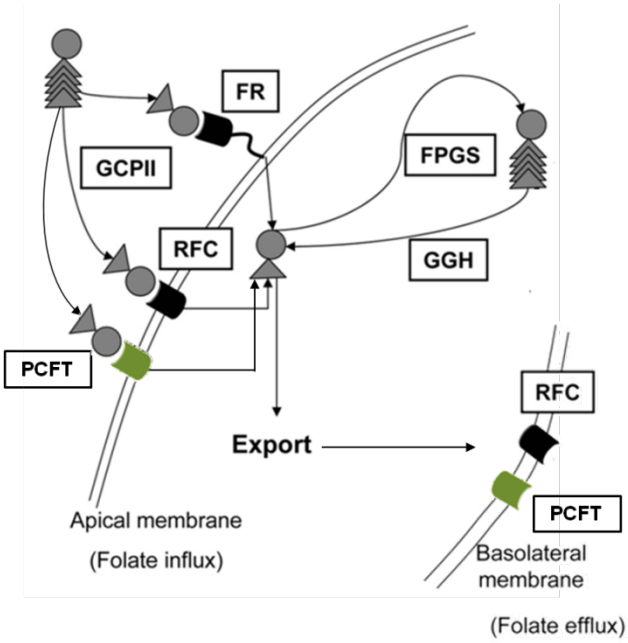

Figure 2.5. Simplified schematic of folate influx and efflux. Adapted and reprinted by permission from the publisher (John Wiley and Sons): [106].

PCFT is an endosomal folate transporter, highly expressed at the apical brush border membrane of the duodenum and proximal jejunum [112, 113]. This receptor is pH-dependent and binds monoglutamated folates at pH 5.8-6.0, and requires the coupling of a proton to facilitate transport [112]. This transporter has similar affinities for reduced (5-methylTHF and 5-formylTHF) and oxidized (FA) folates (K_m=0.5-1.0 μM) [114]. Furthermore, PCFT also mediates endosomal folate export resulting from FRα-mediated invagination of folates [113]. Mutations in the *SLC46A1* gene are associated with hereditary folate malabsorption, an autosomal recessive disease, in which children present with severe folate deficiency resulting in

anemia [115]. Thus, PCFT is the primary transport responsible for maintaining intracellular folate homeostasis.

A fourth and less considerable method of transport is by passive diffusion, which only occurs as a pharmacological effect and is not implicated under normal conditions [110, 111].

Table 2.5. Comparison of three main transport systems for cellular folate uptake and efflux [106, 113].

	RFC	FRα	PCFT
Location	Apical and basolateral membranes of epithelial cells	Apical brush border membrane of epithelial cells	Apical brush border membrane of duodenum and proximal jejunum
Mechanism	Anion-dependent transport of folate influx (and efflux?)	Endocytic process to internalize folates	Low pH and H^+-dependent endosomal uptake. Mediates endosomal folate export from FRα-mediated invagination
Affinities			
5-methylTHF	K_m = 1-5 μM	K_m = 3 nM	K_m = 0.5-1.0 μM
Folic acid	K_m = 100-200 μM	K_m < 1nM	K_m = 0.5-1.0 μM

Once intracellular, the transported folates must be polyglutamated in order to sequester them within the cell. This process is mediated by folylpolyglutamate synthase (FPGS), which elongates the monoglutamated tail, a method of metabolic trapping [109]. This process can be reversed by γ-glutamyl hydrolase (GGH) which cleaves folates into their monoglutamated forms, suitable for cellular efflux [109]. Thus, intracellular folate concentration is modulated of FPGS and GGH activities. These folates are hydrolyzed to dihydrofolate (DHF) which then undergo reduction to tetrahydrofolate (THF) by a catalytic reaction regulated by dihydrofolate reductase (DHFR). THF and 5,10-methyleneTHF is converted to 5-methylTHF by MTHFR using $FADH_2$ (1,5-dihydroflavin adenine dinucleotide) as an electron donor, and released into the portal circulation [1]. The majority of this folate is taken up by the liver, metabolized into polyglutamate forms, and retained or released into the blood or bile. However, a recent study

suggested that the liver is the initial site of folate metabolism, and not the cells of the small intestine [116].

In circulation, folates are monoglutamated and free to enter the glomerulus. They are reabsorbed by FR in the proximal renal tubule or excreted in the urine mainly in the form of its cleavage products, since intact folates are more likely to be found in biliary excretion, which are reabsorbed in the small intestine [110, 111]. Small quantities are detected in fecal excretion however it is difficult to distinguish dietary losses, from losses of folate synthesized by colonic microflora.

2.2.3. Biochemical functions of folate

There are three main active forms of intracellular folate metabolites, THF, 5,10-methyleneTHF and 5-methylTHF, which take part in three main functional roles of intracellular folate metabolites which are highly interconnected processes: 1) nucleotide biosynthesis, 2) regeneration of methionine, and 3) biological methylation (**Figure 2.6**).

2.2.3.1. Nucleotide biosynthesis

THF is twice-methylated by serine hydroxymethyltransferase (SHMT) into 5,10-methyleneTHF using methyl groups from serine. This reaction is reversible with glycine to accept the methyl groups and regenerate serine and THF. In the form of 5,10-methyleneTHF, it can be directed towards purine or pyrimidine synthesis. For purine synthesis, 10-formylTHF is formed and catalyzed by glycinamide ribonucleotide formyltransferase (GARFT), and THF is regenerated.

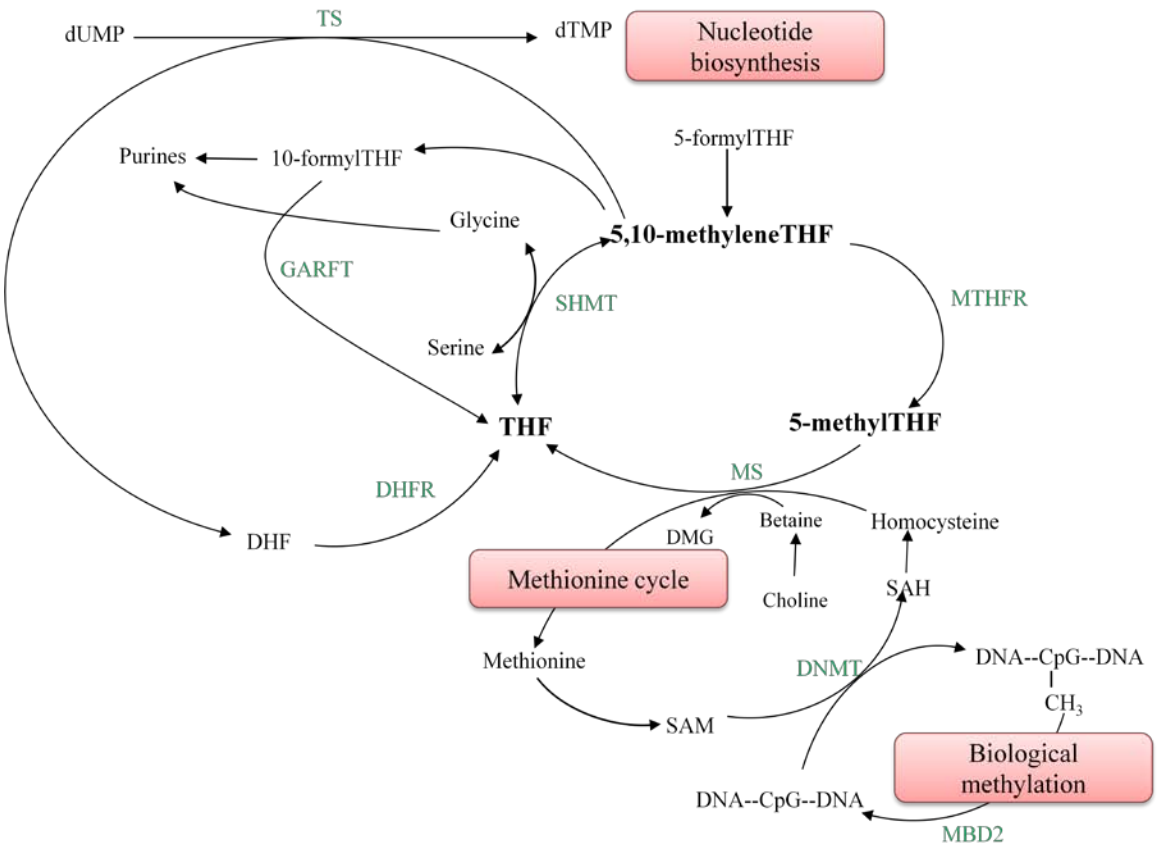

Figure. 2.6. Biochemical functions of folate. Within the cell, folate exists as one of three active metabolites (bolded): tetrahydrofolate (THF), 5,10-methyleneTHF and 5-methylTHF, and have a role in nucleotide biosynthesis, methionine cycle and biological methylation reactions. Dihydrofolate reductase (DHFR) and serine hydroxymethyltransferase (SHMT) are involved in the maintenance of the intracellular folate pool. Thymidylate synthase (TS) and glycinamide ribonucleotide formyltransferase (GARFT) is involved in nucleotide biosynthesis. Methytetrahydrofolate reductase (MTHFR) and methionine synthase (MS) are involved in the methionine cycle. DNA is methylated by DNA methyltransferases (DNMTs) and unmethylated by DNA demethylase (MBD2). DHF=dihydrofolate, dUMP=deoxyuridine-5-monophosphate, dTMP=deoxythymidine-5-monophosphate, DMG=dimethylglycine, SAM=S-adenosylmethionine, SAH=S-adenosylhomocysteine, CpG=cytosine-phosphate-guanine dinucleotide sequence. Adapted and modified by permission from the publisher (John Wiley and Sons): [106].

For thymidine synthesis, 5,10-methyleneTHF and dUMP bind to TS and generate dTMP and

DHF. THF can then be regenerated from DHF by DHFR.

2.2.3.2. Regeneration of methionine

Methionine is regenerated from homocysteine using 5-methylTHF as the methyl donor in a reaction catalyzed by methionine synthase (MS) with methylcobalamin, a derivative of vitamin B_{12} [117, 118]. Regenerated methionine is adenylated to form S-adenosylmethionine (SAM) which is converted to S-adenosylhomocysteine (SAH) when methyl groups are further donated for methylation of DNA, a reaction catalyzed by DNA-methyltransferases (DNMTs). As such, SAM is the primary one-carbon donor for over eighty different biological methylation reactions and is negatively inhibited by rising concentrations of SAH [117-119]. Following this step, SAH is converted to homocysteine, completing the cycle with the regeneration of methionine and THF.

2.2.3.3. Biological methylation

There are two main methods of epigenetic control, DNA methylation, and histone methylation and acetylation, which effectively change gene expression without altering the nucleotide sequence [117, 118]. As the main donor of one-carbon units, SAM concentrations and DNMT activity are important determinants of DNA methylation. Patterns of methylation at cytosines in repetitive CG sequences are heritable and tissue-specific postsynthetic modifications of human DNA [120]. The majority of CpG sites are normally methylated and are sparsely found throughout the genome at exons, non-coding regions and repeat DNA elements [121] (**Figure 2.7**). Methylation of CpG-depleted bulk of the genome effectively controls transcriptional silencing of genes and sequences, which would otherwise compromise the structural integrity of the genome [122]. In addition, global DNA methylation effectively allow for the organization of chromatin in active and inactive states [122]. By contrast CpG-rich sequences, called CpG islands, and are primarily located upstream near promoters, exon 1, or 5’ untranslated regions

[60]. In normal cells, these regions are unmethylated, thereby allowing transcription factors to bind.

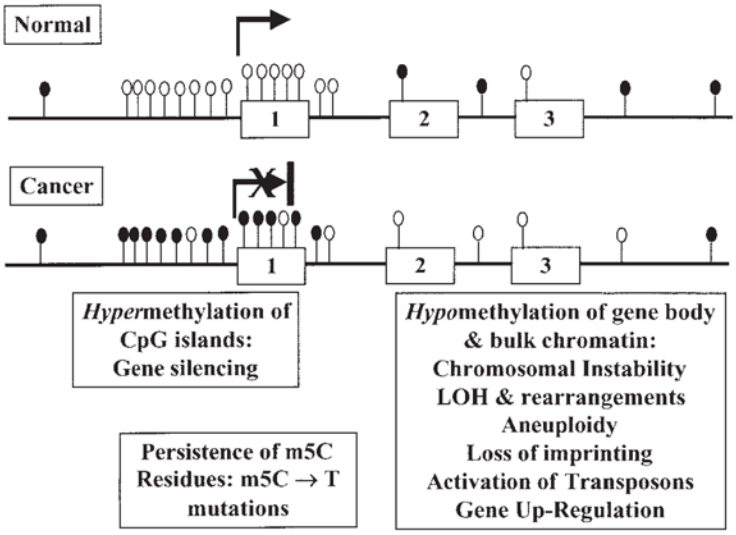

Figure 2.7. Distribution of CpG sequences in the human DNA and methylation patterns in normal (top) and cancer cells. In normal cells, CpG-sparse regions are methylated (dark circles) suppressing transcription of genes which would otherwise compromise genomic stability, while promoter regions are unmethylated (white circles). By contrast in cancer cells, CpG-dense promoter regions are hypermethylated and regions normally methylated throughout the bulk of the genome is now hypomethylated, promoting chromosomal instability, LOH, rearrangements, among others. Numbered boxes indicate exons and lines between them indicate introns. Arrow indicates site of transcription start. Adapted and reprinted by permission from publisher (John Wiley and Sons): [106]

In contrast to methylation of the CpG-depleted bulk of the genome and unmethylated CpG islands in normal cells, cancer cells exhibit a different and distinctive pattern. In cancer cells, there is global hypomethylation and gene-specific hypermethylation, threatening genomic stability and defense against oncogenic factors, respectively. Global hypomethylation of bulk chromatin occur early in carcinogenesis and leads to chromosomal instability, aneuploidy, LOH and activation of genes normally suppressed [123, 124]. Gene-specific methylation in cancer cells occur in promoter sites of genes which are responsible for tumor suppression, cell cycle

control and apoptosis, cellular differentiation, repair of damaged DNA, proliferation, angiogenesis, and metastasis [122].

Folate plays an important role in one-carbon transfers ensuring regeneration of methionine and the consequent availability of SAM for biological methylation reactions. Both preclinical and clinical studies add to the growing body of evidence of the role of folate in modulating DNA methylation. In clinical studies where subjects received a low dose of folate (up to 118 μg/d) for up to nine weeks, subjects exhibited a decrease in genomic methylation in leukocytes [125, 126]. As well, FA supplementation of 5 and 10 mg/d in patients with a history of colonic adenoma, demonstrated an increase in genomic methylation in rectal mucosa at both three and six months after intervention began [127-129].

2.2.3.4. Single nucleotide polymorphisms (SNPs) in folate metabolism

Several genetic polymorphisms and variants among important enzymes are involved in folate metabolism. Among them, SNPs in the *MTHFR* gene have received the most attention [130]. MTHFR is critical in maintaining intracellular folate homeostasis as it regulates the separate concentration of folates reserved for nucleotide synthesis and one-carbon metabolism. MTHFR catalyzes the irreversible conversion of 5, 10-methyleneTHF to 5-methylTHF, the primary precursor of SAM. A common mutation involves a cytosine to thymine substitution at position 677, causing an amino acid substitution from alanine to valine [130]. The C677T SNP decreases MTHFR activity and increases thermolability of the enzyme, effectively reducing the availability of 5-methylTHF to regenerate methionine [130]. As such, individuals bearing the 677TT mutation in combination with a low folate status have higher plasma homocysteine concentrations, compared to individuals bearing the wildtype 677CC [131]. Caucasian and Asian populations have a higher rate of this mutation at 12-15% who are homozygous 677TT and up to

50% who are heterozygous 677CT [130]. In North America, there is an allele frequency of approximately 35% in the general population [131]. An accumulating body of evidence suggests that this SNP can affect risks of some diseases [132-134]. Similar to folate deficiency, it has been implicated in the risk of cardiovascular disease, neural tube defects (NTDs) and other adverse pregnancy outcomes, Down's syndrome, neuropsychiatric disorders and cancer [130, 135-138].

A second common SNP in the MTHFR gene involves an adenine to cytosine substitution at position 1298, causing an amino acid substitution from glutamine to alanine [139]. Similar to the C667T polymorphism, A1298C decreases enzyme activity but does not affect thermolability [139]. Though an allele frequency similar to C677T has been reported (up to 33%), evidence of the relationship between plasma homocysteine concentrations and the folate made available are inconsistent [139-141].

Several variations have recently been described in DHFR, including promoter polymorphisms, the 19-base pair deletion allele and variations in 3'-untranslated region. These polymorphisms are functional and affect DHFR mRNA levels through many mechanisms. The most extensively studied *DHFR* polymorphism is the 19-base pair insertion to deletion in the first intron. This polymorphism causes low plasma folate and elevated homocysteine due to changes in mRNA levels [142-144]. There is evidence that individuals with the *del/del* genotype have significantly increased DHFR mRNA levels, and subsequently DHFR expression, such that there exists maternal protection against NTDs by increasing the amount of DHFR available to reduce DHF to THF [143, 145]. However, this has been challenged by Kalmbach *et al* suggesting that the *del* allele is associated with a decreased capacity to reduce FA [146]. The role of *DHFR* polymorphisms in NTD risk remains to be determined. Recently, the potential relationship between germline variants in methyl-group metabolism genes and promoter CpG

island methylation in CRC tumors has been demonstrated [147]. Given that many chemotherapeutic regimens are antifolate-based, *DHFR* polymorphisms and the effect on cancer treatment response has been studied. Higher DHFR activity and THF accumulation can challenge the cytotoxic effect of antifolates such as MTX, and thereby reduce treatment efficacy. More specifically, variations in the 3'-untranslated region result in higher mRNA and protein levels of DHFR, leading to resistance to MTX [148]. DHFR gene variations are associated with changes in protein levels, thereby affecting intracellular folate concentrations, and could be an important determinant in susceptibility to disease and treatment outcomes [149].

A number of other SNPs in folate metabolism have been investigated. Genes involved in absorption (GCPII), uptake (FR, RFC), cellular retention (FPGS) and export (GGH), nucleotide biosynthesis (TS, SHMT, GARFT), regeneration of methionine (MS) have been identified [138]. A recent pharmacogenetic study by Jennings *et al* suggests that a polymorphism in the 5' untranslated region of TS resulting in lower protein expression was more responsive to chemotherapy in patients with CRC, whereas there was no significant effect of either MTHFR SNPs to response to treatment [150].

As such, identifying functional polymorphisms and elucidating the effects have facilitated the understanding that certain populations may be greater impacted by disease states, such as individuals with low folate status and high homocysteine concentrations. Further investigations will undoubtedly aid in understanding its bearing on affected individuals, in addition to possible enhancement of current treatment options, towards personalized medicine.

2.3 Folate and Health

A higher folate status attained through dietary intake is generally associated with a lower risk of disease. A large number of epidemiological studies report an association between low

folate status and increased risk of megaloblastic anemia, neural tube defects and other adverse birth outcomes [106]. Inadequate folate intake cause disruptions in erythrocyte formation, such that cells appear larger in size due to their inability to produce DNA quickly enough to divide at the appropriate time, and are insufficient in hemoglobin content. Closer examination of megaloblastic anemia also show irregularities in cells involved in immune function, and the presence of hypersegmented neutrophils. Long-term exposure to low folate-induced anemic conditions result in fatigue, nerve damage and peripheral neuropathy, mental confusion and depression, heart palpitation and other behavioural disorders. FA supplementation at moderate doses reverse these effects and deliver adequate folates necessary for erythrocyte formation. However, there is also a growing body of evidence that FA supplementation may be associated with several potentially harmful effects, such as decreased natural killer cytotoxicity, cognitive decline, genetic selection of disease alleles, resistance to antifolate based chemotherapy, anti-inflammatory drugs, anti-seizure treatments, epigenetic instability, and some cancers [106, 117, 151-153]. It is clear that folate plays a more complicated role in health promotion and disease prevention, than once believed.

2.3.1. Rationale for folic acid fortification

There were several epidemiologic and intervention studies suggesting the protective role of FA against NTDs, but it was convincing evidence in the early 1990s by the Medical Research Council (MRC) Vitamin Study that served as the primary force behind the fortification of foods with FA [154]. During the periconceptional period, a higher demand of folate is required to support the accelerated rate of cellular growth and differentiation. This study was a multi-centered, double blinded, randomized control trial with pregnant women considered high-risk of having a NTD-affected pregnancy. Daily intervention with 4 mg of FA showed a near 72%

protective effect, resulting in an early termination of the study [154]. Consequently, both the United States and Canadian governments initiated fortification of all white flour, cornmeal and pasta in 1996 and 1998, respectively; however voluntary fortification by the industry began as early as 1992. In Canada, 150 µg FA per 100 g of enriched flour and uncooked cereal grains was added, intended to provide an additional 100-200 µg FA daily. This dosage was thought to supplement a diet by 30-60% the average folate intake among women of child-bearing age, and pose no adverse effects in the overall population [155].

2.3.2. Result of folic acid fortification in the prevention of neural tube defects

Unequivocally, widespread FA fortification to lower the prevalence of NTDs is considered one of the most successful public health initiatives in the United States and Canada. Numerous studies from both countries show a significant reduction in NTDs [2, 156-158]. A population-based study in Canada shows that the prevalence of NTDs decreased from 1.58 per 1000 births before fortification, to 0.86 per live births during the full fortification period, a 46% reduction (95% CI, 40-51) [2]. In 2007, MOTHERISK, a research group at the Hospital for Sick Children in Toronto jointly released updated clinical guidelines with the Society of Obstetricians and Gynecologists of Canada (SOGC) recommending a diet rich in folate and a prenatal vitamin at least three months prior to conception through pregnancy, and as long as breastfeeding continues [159]. MOTHERISK further emphasizes that unless clinicians can be assured that pregnant women will reliably use prenatal supplements, the supplements should be combined with 5 mg FA [160]. This is substantiated by a study in 2001 where authors stated that at a daily dose of 5 mg FA would reduce NTD risk by 85% [161]. In 2009, the SOGC and Health Canada released an update to their guidelines for prenatal nutrition suggesting that since the majority of

pregnancies are unplanned, that all women capable of becoming pregnant take a daily multivitamin containing 400 µg FA [107].

2.3.3. Intake and blood levels in the post-fortification era

NHANES data show median erythrocyte levels of persons over 4 years have increased by 60% from 174 ng/mL to 276 ng/mL, between 1988-1994 and 1999-2000, and most recent data (2004-2006) show levels of 266 ng/mL [162]. Canadian population data of serum folate concentrations show similar trends where there was a steady increase since fortification and a recent plateau-effect at increased levels [163] (**Figure 2.8**). Though this public health initiative was intended to provide an additional 100-200 µg per day, a recent study of the 95 most commonly purchased fortified foods shows that FA levels are on average 151% ± 63 of what is reported in the Canadian Nutrient File of the product [164]. Overfortification by the industry ensures adequate levels throughout the shelf life of the product, however it exposes the population to levels higher than intended. A recent study of the Canadian Health Measures Survey (CHMS), authors report that folate deficiency (erythrocyte folate <305 nmol/L) is evident in less than 1% of the population and in fact, 40% have high folate concentrations (erythrocyte folate >1360 nmol/L), a level chosen reflecting the 97% percentile from the NHANES (1999-2004) data [12] (**Figure 2.9**).

Though among women of childbearing age, 22% showed erythrocyte folate concentrations below 906 nmol/L, the level considered optimal for maximal protection against NTDs [12], it is unknown whether these women are interested in becoming pregnant or capable of pregnancy.

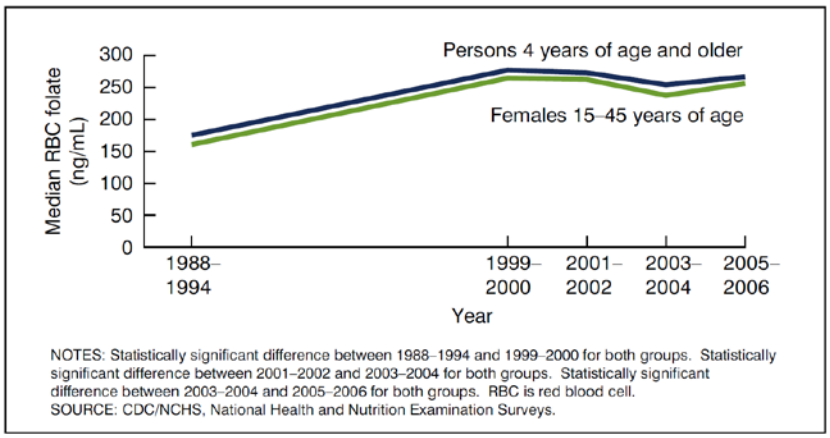

Figure 2.8. Median erythrocyte folate (ng/mL) of the U.S. population, according to most recent NHANES data (2005-2006). Open access publication, permission for adaptation and/or reprint not necessary: [162].

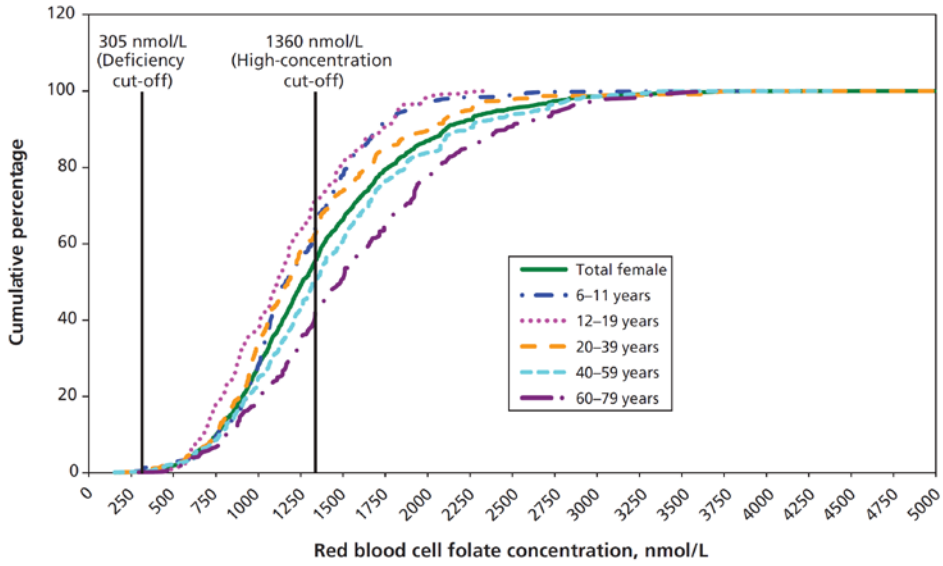

Figure 2.9. Cumulative percentile distribution of erythrocyte folate concentrations, as reported in the Canadian Health Measures Survey, 2007-2009. Folate concentrations for deficiency (<305 nmol/L) and high folate concentrations (>1360 nmol/L) are indicated by vertical lines. Female data is shown, however male data shows similar trends. Copied under license from the Canadian Medical Association and Access Copyright. Further reproduction prohibited: [165].

2.3.4. Dietary supplement use

2.3.4.1. Among the healthy population

The use of a daily multivitamin or single-vitamin capsule to enhance one's diet has become a ubiquitous and habitual practice among North Americans. In fact, data from NHANES and the Canadian Community Health Survey (CCHS) provide evidence that up to 50% of adults regularly use a dietary supplement [4, 5]. Data from the United States further state that 70% of the population over 70 years of age also takes at least one dietary supplement per day [5]. A number of studies show that individuals amongst the highest percentile of total folate and FA intake have greater contributions from dietary supplements [166] (**Figure 2.10**). In fact, 40% over the age of 60 years have detectable levels of unmetabolized FA (UMFA) which persist after fasting [6, 167]. Regular use is correlated with age, race, socioeconomic status and education, where users are predominantly over 50 years of age, non-Hispanic whites, and middle to upper class with more than a high-school education [5]. Recent studies show that supplement use is the most significant predictor of folate status, not diet [7, 166, 168].

2.3.4.1. Among cancer patients and survivors

Vitamin and mineral supplement use among cancer patients is widespread although its long-term beneficial or harmful effects are largely unknown. The highest prevalence of use is among Caucasian, female breast cancer patients and survivors who are of middle to upper SES [9]. A recent systematic review [9] summarized 32 studies between 1999 and 2006, and found that regular supplement ranged from 64 to 81% among cancer survivors, which is a higher prevalence than the general population of 50% [11, 169-171]. It is reported that up to 68% of physicians are unaware of their patients' supplement use [9]. Furthermore, over 50% of CRC patients report taking a daily supplement during the course of their treatment [10, 172]. In fact, a

large proportion adopted this new habit upon diagnosis [173]. Patterson *et al* puts forth that news of life-threatening health events such as cancer diagnosis, can trigger psychological distress and prompt individuals to undertake health promotion activities in attempt to decrease further disease progression [11]. In fact, motivation to improve dietary and exercise behaviours, and undertake other risk factor interventions is most common among the recently diagnosed and survivors [174]. A recent study of the Colon Cancer Family Registry reported that only 35.4% of patients regularly used supplements before diagnosis, and this number increased to over 55% following diagnosis [173]. The National Cancer Institute strongly discourages patients undergoing treatment to take vitamin or mineral supplements, unless otherwise recommended by their physician [175]. It is unknown whether supplementation can enhance, have a null effect or interfere with their treatment.

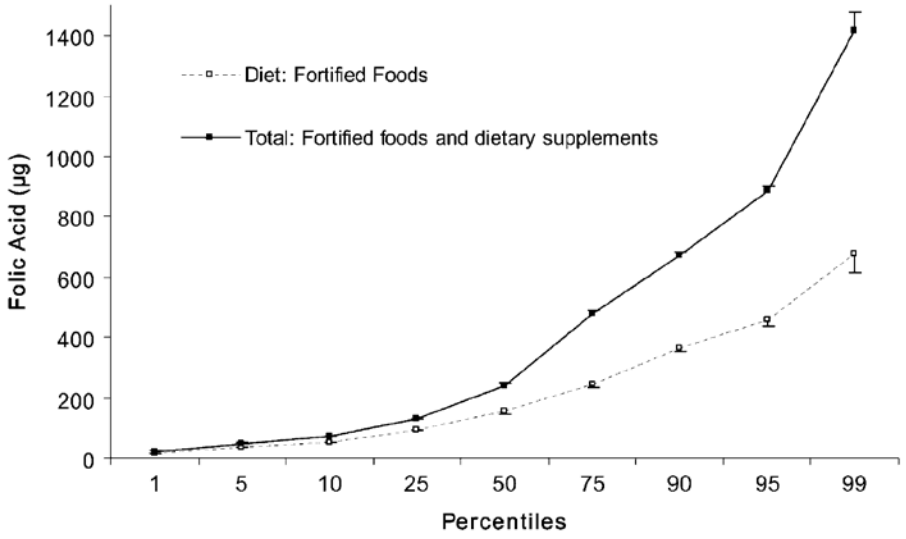

Figure 2.10. Mean (±SE) percentiles of dietary and total FA intake in the United States (NHANES 2003-2006). Dietary FA is from fortified foods, and total FA includes fortified foods and dietary supplements. As total FA intake increases, the proportion contributed to by supplement use increases almost exponentially. Adapted and reprinted by permission from the publisher (American Society for Nutrition): [6].

2.3.5. Potential adverse effects of high folate and folic acid intake in the postfortification era

The daily UL remains 1 mg FA/day, but there lacks a consensus on safe limits of plasma or erythrocyte folate concentrations. Though the evidence for the prevention of NTDs is unequivocal, FA supplementation may present other adverse health outcomes. Particularly among the North American population where dietary supplement use is abundant, in addition to a food supply rich in FA, it is imperative to elucidate the complex role of folate in health and disease states. There is a growing body of evidence that FA supplementation at high doses has adverse outcomes, including masking of vitamin B12 deficiency, accelerated cognitive decline in those with low vitamin B12, decreased natural killer cytotoxicity, resistance to certain medications (anti-inflammatory drugs, anti-seizure medications, anti-folate chemotherapy), and promoting the progression of existing preneoplastic lesions [151, 176].

Briefly, vitamin B12, also called cobalamin, provides an essential cofactor of MS for the effective conversion of 5-methylTHF and homocysteine to THF and methionine. Individuals with B12 deficiency present with fatigue and classical signs of anemia, however, high levels of FA can bypass the metabolic block in nucleotide synthesis, allowing cell division to continue, and ultimately mask signs of low B12-induced anemia [151]. In addition, epidemiological evidence suggest that elderly people with high folate-low B12 status are at a greater than 70% increase risk of cognitive impairment [152]. A prospective study by Morris *et al* provides evidence from people aged 65 years or greater, and further states that the rate of decline was highest among those taking an additional 400 μg FA supplement [177]. Though exact mechanisms contributing to cognitive decline are unknown, high intracellular DHF concentration is a potent inhibitor of both TS and MTHFR, and results in impaired DNA synthesis, inhibit the formation of 5-methylTHF and decrease regeneration of methionine [178, 179].

Other potential harmful effects have been speculated in population exposed to FA in fortified foods. In the post-fortification period, detectable levels of UMFA have been found in approximately 40% of older adults among the U.S. population following fasting [167]. A small clinical study providing subjects with a 400 μg FA daily supplement preload and bread slices fortified with 100, 200 and 400 μg FA, has reported detectable UMFA when a total of 800 μg/day FA was consumed [180]. In a study among postmenopausal women, there was an inverse association between the presence of plasma UMFA and natural killer cell cytotoxicity [176]. It is unknown whether circulating UMFA has potentially harmful effects; some suggest that there are mechanisms by which the human body is able to adapt to high FA intake [181].

Perhaps the most studied relationship between folate and health is cancer risk and the effect of folate on carcinogenesis. Cancers of the lung [182, 183], prostate [184], esophagus [185], stomach [185], pancreas [185, 186], breast [187, 188], colorectum (**Tables 2.6 to 2.11**), ovaries [189], and cervix [190], have been investigated in relation to folate intake and whether FA supplementation could be beneficial as a therapeutic intervention. CRC is without a doubt the most studied cancer and provides the most compelling epidemiologic, clinical and animal evidence supporting the role of folate in carcinogenesis.

2.4. Folate and colorectal cancer

With the accumulating knowledge of the biological functions of folate, a number of epidemiological and experimental studies have explored the relationship between folate and cancer. To date, there have been 31 case-control (**Tables 2.6 and 2.7**), 13 prospective cohort (**Table 2.8**) and 18 randomized controlled trials (**Tables 2.9 to 2.11**).

2.4.1. Current evidence

Though epidemiological evidence has been conflicting, studies exploring the relationship between folate intake and CRC risk have generally shown an inverse correlation, where the greater the intake, the lower the risk. Overall, there is a 20-40% decreased risk of CRC and adenoma when those with highest intake of folate are compared to those with lowest intake of folate [14, 191-194]. The relationship between erythrocyte levels of folate and the risk of CRC and adenoma is less well defined than that between dietary intake and risk of CRC and adenomas [13, 14, 195].

The largest cohort study to date is the NIH-AARP Diet and Health Study and consists of 525 488 individuals [196]. This study included U.S. adults aged 50-71 years with a mean follow-up time of 8.5 years, and authors reported a significant inverse trend with higher dietary, supplement and total folate intake [196]. Similarly, Giovannuci *et al* [197] reported that higher total folate intake was associated with a lower risk following a 15 year follow-up (RR=0.69, 95% CI: 0.52-0.93, p=0.01). There was no beneficial effect of multivitamins among users of less than 15 years (RR=0.82, 95% CI: 0.56-1.20), however there was a lower risk among multivitamin users of over 15 years (RR=0.21, 95% CI: 0.05-0.84, p=0.003) [197].

A meta-analysis by Sanjoaquin *et al* included nine case-control and seven cohort studies and succinctly divided studies by type and the measurement of folate used [198]. The analysis shows that, among cohort studies, there is a significant 25% lower risk of CRC among those in the highest category of dietary folate intake compared to those in the lowest category [198]. Among studies which investigated total folate intake, there was a non-significant 5% decrease in risk [198]. These authors reported that case-control studies showed similar trends, however there was significant heterogeneity among studies [198]. These findings are supported by Kim *et al*

who concludes that higher folate intake is associated with an decreased risk, with a 2% risk reduction for every 100 μg/day in a pooled analysis of thirteen prospective cohort studies [199]. One hypothesis of the chemopreventive role of folate in (pre)neoplastic tissue is its involvement in DNA methylation. Since cancer cells are understood to undergo genomic hypomethylation early in carcinogenesis, folate is hypothesized to suppress, and even reverse, hypomethylation. However, in a more recent meta-analysis, Kennedy *et al* included eighteen case-control and nine cohort studies of colon, rectal and colorectal cancers [200]. These authors reported a non-significant reduced risk in the high folate intake category compared to the low folate intake category in regards to dietary folate intake in analysis of both case-control and cohort studies (**Figure 2.11**). However, when total folate intake was analyzed, authors found a statistically significant risk reduction (OR=0.85, 95% CI: 0.74-0.99) [200] (**Figure 2.12**).

Table 2.6. Summary of case-control studies of folate intake and colorectal cancer risk*¶

Reference	Country/study Subject characteristics	No. of cases/controls	Folate source	OR (95% CI)	P-value
Freudenheim *et al* [201]	United States Caucasian	428/428	Dietary folate <210 vs. >340 µg/d	M: 1.03 (0.56-1.89) F: 0.69 (0.36, 1.30)	N.S. N.S.
Ferraroni *et al* [202]	Italy 20-74 years old; median age 62 years	828/1189	Dietary folate <162 vs. >227 µg/d	0.52 (0.40-0.68)	P<0.05
White *et al* [203]	USA 30-62 years old	M: 251/233 F: 193/194	Supplemental folate 0 vs. ≥400µg/d	M: 0.59 (0.34-1.01) F: 0.44 (0.24-0.80)	P=0.04 P=0.007
Benito *et al* [204]	Majorca 50-75 years old	286/296	Dietary folate <141 vs. >222.3 µg/d	0.27 (N.A.)	P<0.01
Boutron-Ruault *et al* [205]	France 30-79 years old	171/309	Total folate <218 vs. >402.5 µg/d	1.0 (0.5-2.0)	N.S.
Slattery *et al* [206]	United States 30-79 years old	1993/2410	Dietary folate M: <120 vs. >210 µg/d F: <130 vs. >230 µg/d	1.20 (0.90-1.60) 0.90 (0.62-1.30)	N.S. N.S.
Le Marchand *et al* [207]	United States The Multiethnic Cohort Study 45-75 years old	822/2021	Total folate <297 vs. >2430 µg/d Dietary folate <252 vs. >406 µg/d	0.80 (0.58-1.10) 0.90 (0.62-1.30)	N.S. N.S.
Jiang *et al* [208]	China	53/343	Dietary folate <115.64 vs. >172 µg/d	0.91 (0.70-1.19)	N.S.
Kune *et al* [209]	Australia The Melbourne CRC Study	715/727	Dietary folate <246 vs. 1367 µg/d	1.24 (0.81-1.89)	N.S.
Lightfoot *et al* [210]	United Kingdom 45-80 years old	124/128	Total folate 267 vs. >397 µg/d	1.08 (0.78-1.50)	N.S.
Sharp *et al* [211]	United Kingdom	255/398	Total folate <263.9 vs. >348.6 µg/d	1.37 (0.80-2.36)	N.S.
Kim *et al* [212]	Korea 30-79 years old	596/509	Dietary folate ≤179 vs. >270.2 µg/d Total folate ≤183.6 vs. >297.5µg/d	0.47 (0.32-0.69) 0.64 (0.45-0.92)	P<0.001 P=0.025
Kim *et al* [213]	Korea 30-79 years old	787/656	Total folate <209.69 vs. >282.72 µg/d	0.64 (0.49-0.84)	P=0.002

Eussen *et al* [214]	Europe EPIC Study	1367/2325	Plasma folate <7.6 vs. >18.3 nM/L	RR=0.94 (0.74-1.20)	N.S.
Terry *et al* [215]	Canada/Canadian National Breast Screening Study 40-59 years old; female	295/5334	Total folate ≤233 vs. >367 µg/d	IRR=0.60 (0.40-1.10)	N.S.
Kato *et al* [216]	Women's Health Study ≥ 45 years old; female	105/523	Serum folate ≤12.23 vs. ≥ 31.04 nM/L Total folate ≤224 vs. ≥ 626 µg/d	0.52 (0.27-0.97) 0.88 (0.46-1.69)	P=0.04 N.S.
Otani *et al* [217]	Japan 40-69 years	375/750	Plasma folate <5.6 vs. >8.6 ng/mL	M: 0.86 (0.45-1.60) F: 1.00 (0.56-1.90)	N.S. N.S.
Glynn *et al* [218]	Finland ATBC Cancer Prevention Study 50-69 years; male smokers	144/276	Serum folate ≤2.9 vs. >5.2 ng/mL	0.51 (0.20-1.3) 4.79 (1.36-16.93)[+]	N.S. P<0.05
Satia-Abouta *et al* [219]	United States/North Carolina 30-80 years; Caucasian (C) and African American (AA)	White: 337/596 AA: 276/400	Total folate <196 vs. >741 µg/d	C: 0.8 (0.5-1.2) AA: 0.9(0.5-1.6)	N.S. N.S.
Van Guelpen *et al* [220]	Sweden/Northern Sweden Health and Disease Cohort	226/437	Plasma folate <5.7 vs. >13 nmol/L	3.87 (1.52-9.87)	P=0.007
Baron *et al* [221]	USA Median age 60 years old	260/449	Dietary folate <214 vs. >388 µg/d Total folate <243 vs. >391 µg/d	0.94 (0.53-1.67) 1.11 (0.69-1.78)	N.S. N.S.
Meyer *et al* [222]	USA 30-62 years	M: 238/224 F: 186/190	Total folate M: <151 vs. >281 µg/d F: <131 vs. 276 µg/d	1.00 (0.81-1.24) 0.81 (0.66-1.00)	N.S. P<0.05
Pufulete *et al* [223]	United Kingdom	28/76	Total folate <260 vs. >348 µg/d	0.09 (0.01-0.57)	P=0.01
Levi *et al* [224]	Switzerland 27-74 years old	119/491	Total folate <173 vs. >403 µg/d	1.54 (0.80-3.1)	N.S.

******P value for inverse trend; N.S.=non-significant; OR=odds ratio; RR=relative risk; IRR=incidence rate ratio; total folate includes folate from foods and supplements

¶ EPIC=European Prospective Investigation into Cancer and Nutrition, ATBC= α-tocopherol β-carotene

[+] In the α-tocopherol β-carotene study [218], men with high alcohol, low folate, low protein at higher risk of CRC than low alcohol, high folate, high protein

Table 2.7: Summary of case-control studies of folate intake and adenoma risk*¶

Reference	Country/study Subject characteristics	No. of cases/controls	Folate source	OR (95% CI)	P value
Pufulete *et al* [223]	United Kingdom	35/76	Total folate <282 vs. >359 µg/d	0.98 (0.30-3.22)	N.S.
Han *et al* [225]	USA PLCO Study 55-74 years	1331/1501	Dietary folate ≤262 vs. >466 µg/d	1.46 (1.17-1.82)	P<0.001
Giovannucci *et al* [192]	USA NHS, HPFS	M: 331/9159 F: 564/15420	Total folate M: <241 vs. >847 µg/d F: <166 vs. >711 µg/d	0.63 (0.41-0.98) 0.66 (0.46-0.95)	P=0.03 P=0.04
Bird *et al* [226]	USA 50-75 years	M: 180/189 F: 152/161	Erythrocyte folate <165 vs. >315 ng/mL Plasma folate ≤3 vs. ≥16.9 ng/mL Total folate intake <242 vs. >576 µg/d	M: 0.47 (0.24-0.90) F: 1.26 (0.65-2.43) M: 0.65 (0.45-0.95) F: 0.95 (0.69-1.30) M: 0.70 (0.36-1.34) F: 1.47 (0.73-2.95)	P=0.02 N.S. P=0.04 N.S. N.S. N.S.
Tseng *et al* [227]	USA	M: 105/165 F: 131/245	Total folate <130.2 vs. >398.0 µg/d	M: 0.84 (0.29-2.43) F: 0.39 (0.15-1.03)	N.S. N.S.
Boutron-Ruault *et al* [205].	France 30-79 years old	1: <10mm adenoma (n=154) 2: large adenoma (n=208) 3. polyp-free (n=426)	Total folate <218 vs. >402.5 µg/d	1 vs.3: 0.5 (0.3-1.0) 2 vs.1: 0.9 (0.4-1.9) 2 vs.3: 0.5 (0.3-1.0)	P=0.03 N.S. P=0.04
La Vecchia *et al* [228]	Italy	1225/4154	Dietary folate <246 vs. >422 µg/d	0.83(0.6-1.1)	N.S.
Benito *et al* [204]	Majorca	101/242	Dietary folate <146 vs. >227 µg/d	0.27 (N.A.)	P<0.01

*****P value for inverse trend; N.S. =non-significant; OR=odds ratio; total folate includes folate from foods and supplements

¶ PLCO = Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial, NHS = Nurses' Health Study, HPFS = Health Professionals' Follow-Up Study

Table 2.8. Summary of prospective cohort studies of folate intake and colorectal cancer risk*¶

Reference	Study/Subjects	No. of cases	Folate Source	RR (95% CI)	P value
Su *et al* [229]	NHANES I Epidemiologic Follow-up Study	219	Dietary folate ≤103.3 vs. >249 µg/d	M: 0.40 (0.18-0.88) F: 0.74 (0.36-1.51)	P=0.03 N.S.
Giovannucci *et al* [191]	Health Professionals Follow-up Study/51 529 men 40-75 years	205 6 y follow up	Dietary folate <269 vs. >646 µg/d	0.86 (0.50-1.47)	N.S.
Zhang *et al* [230].	WHS/39 876 women >45 years	220 10.1 y follow-up	Total folate <259 vs. ≥614 µg/d Dietary folate <244 vs. ≥385 µg/d Dietary folate Ŧ <244 vs. ≥385 µg/d	1.16 (0.76-1.79) 0.67 (0.43-1.03) 0.46 (0.26-0.81)	N.S. N.S. P=0.02
Konings *et al* [231].	Netherlands Cohort Study/120 852 55-69 years	1171 7.3 y follow up	Total folate <168 vs. >266 µg/d	M: 0.73 (0.46-1.17) F: 0.68 (0.39-1.20)	P=0.03 N.S.
Gibson *et al* [196].	NIH-AARP Diet & Health Study/525 488 50-71 years	7212 8.5 y follow up	Dietary folate <200 vs. ≥600 µg/d FA supplement 0 vs. >400 µg/d Total folate <200 vs. >900 µg/d	0.81 (0.67-0.97) 0.82 (0.72-0.92) 0.70 (0.58-0.84)	P=0.003 P<0.001 P<0.001
Giovannucci *et al* [197].	NHS/88 756 women 33-55 years	442 15 y follow up	Total folate ≤200 vs. >400 µg/d Multivitamin use (<15 years) ≤200 vs. >300 µg/d Multivitamin use (>15 years) ≤200 vs. >300 µg/d	0.69 (0.52-0.93) 0.82 (0.56-1.20) 0.21 (0.05-0.84)	P=0.01 N.S. P=0.003
Harnack *et al* [194].	Iowa Women's Health Study/41 836 55-69 years	598	Total folate ≤32.1 vs. >2555.2 µg/d	1.12 (0.77-1.63)	N.S.
Flood *et al* [193].	Breast Cancer Detection Demonstration Project Follow-up Cohort/45 264 women	490 8.5 y follow up	Total folate <188 vs. >633 µg/d Dietary folate <142 vs. >272 µg/d	1.01 (0.75-1.35) 0.86 (0.65-1.13)	N.S. N.S.
Ishihara *et al* [232].	JPHC/81 184	526 >5 y follow up	Dietary folate M: <246 vs. >461 µg/d F: <267 vs. >514	HR=1.20 (0.85-1.71) HR=1.33 (0.85-2.09)	N.S. N.S.
Kabat *et al* [233].	Canadian National Breast Cancer Screening Study/ 89 835 women 40-59 years	617 16.4 y follow up	Dietary folate <237 vs. >374 µg/d	0.89 (0.67-1.17)	N.S.

De Vogel *et al* [234].	Netherlands Cohort Study/120 852	2349 13.3 y follow up	Total folate M: <160.8 vs. >297.2 µg/d F: <139.0 vs. >267.3 µg/d	 0.87 (0.65-1.15) 1.25 (0.89-1.76)	 N.S. N.S.
Larsson *et al* [235].	Swedish Mammography Cohort/66 651 women 40-75 years	805 14.8 y follow up	Dietary folate <150 vs. ≥212 µg/d	 0.80 (0.60-1.06)	 N.S.
Schernhammer *et al* [236].	NHS/121 701 women 33-55 years	399 22 y follow up	Total folate <200 vs. ≥400 µg/d	All cases: 0.80(0.61-1.06) P53 +ve: 0.54 (0.35-0.83) P53 –ve: 1.05 (0.73-1.51)	N.S. P=0.008 N.S.

*****P value for inverse trend; N.S.=non-significant; RR=relative risk; total folate includes folate from foods and supplements

¶ NHANES = The National Health and Nutrition Examination Survey, JPHC = Japan Public Health Cohort, NHS = Nurses' Health Study, WHS = Womens Health Study, M=males, F=females

Ł excluding supplement users

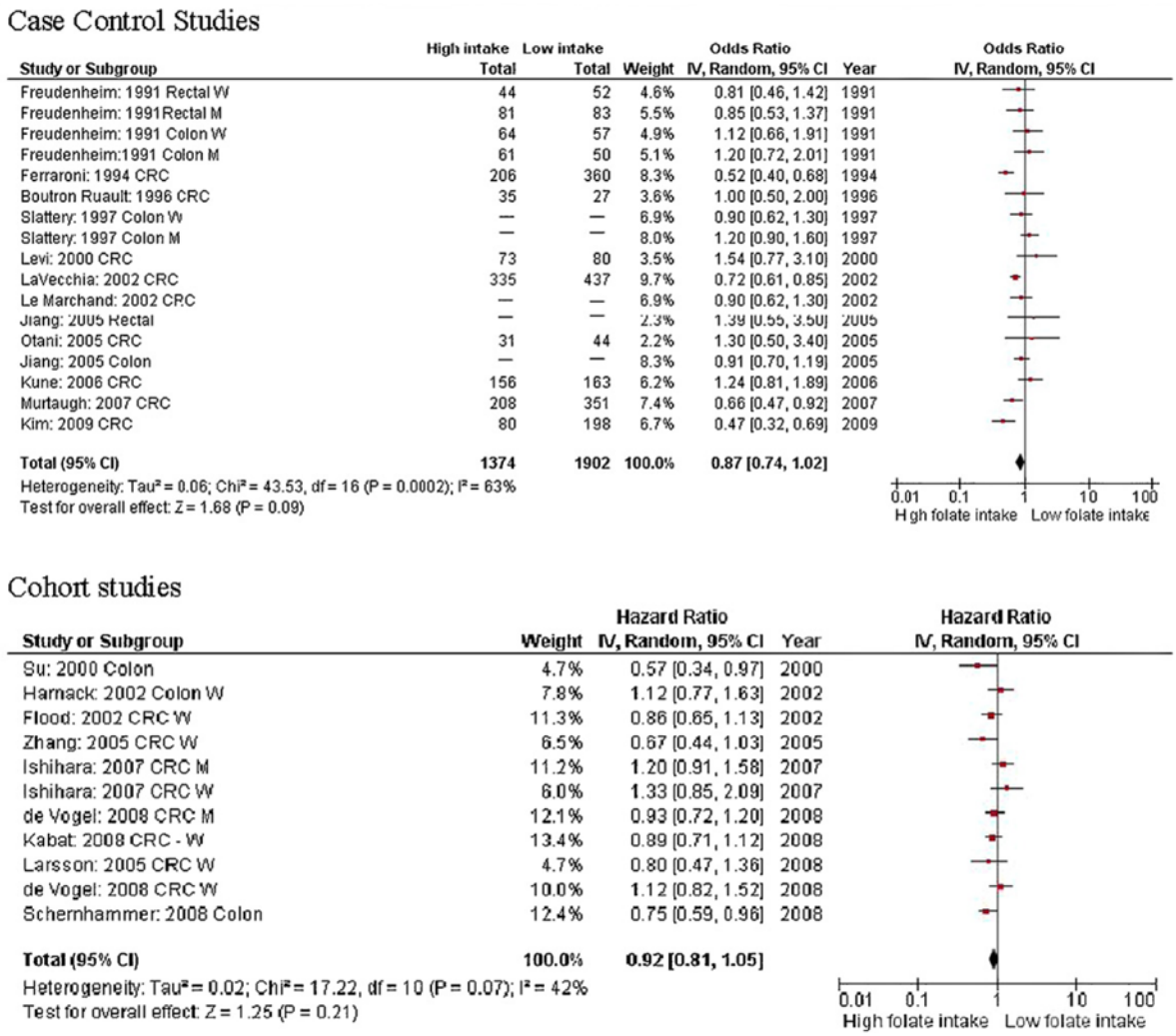

Figure 2.11. Summary of dietary folate intake and case-control and cohort studies in the risk of colon, rectal and colorectal cancers in men (M) and women (W). Adapted and reprinted by permission from publisher (Elsevier): [200].

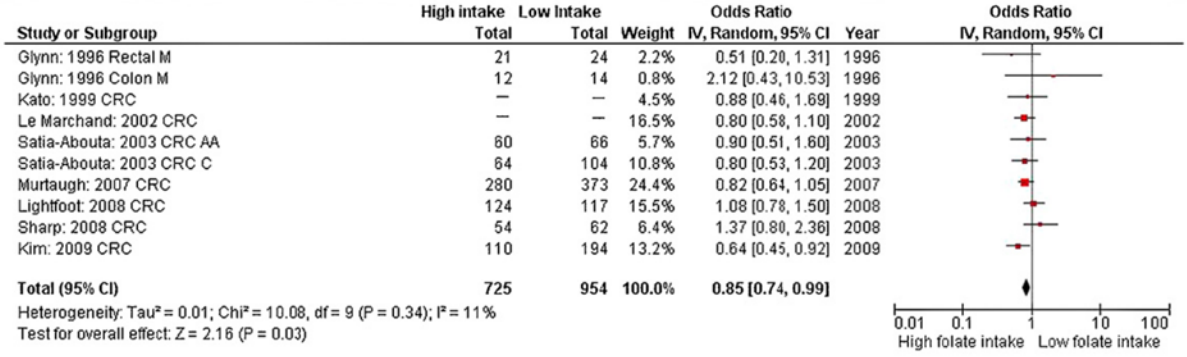

Figure 2.12. Summary of total folate intake and case-control studies in the risk of colon, rectal and colorectal cancers in men (M) and women (W). Adapted and reprinted by permission from publisher (Elsevier): [200].

2.4.1.1. Randomized control trials

Patients recruited to CRC chemoprevention trials are those who have had a recent adenoma or cancer removed and are considered malignancy-free, yet remain at risk. There are a number of intervention studies with FA supplementation in the prevention of adenomas, and CRC exist. **Tables 2.9 to 2.11** summarize randomized control trials investigating FA supplementation on biomarkers of colorectal carcinogenesis, adenoma recurrence, and CRC incidence as a secondary endpoint, respectively. Overall, in studies using FA supplementation as a chemopreventive agent, as assessed by biomarkers of CRC, evidence suggest that higher doses of supplementation result in DNA methylation [127-129, 237]. Since folates mediate one-carbon metabolism in biological methylation reactions, it is evident that methylation can be modulated by diet in studies as short in duration as six months [127-129, 237]. However, when recurrence of adenomas are considered as a surrogate marker for CRC risk, the relationship between FA supplementation and risk is less well defined [238-242]. In the Aspirin/Folate Polyp Prevention Study, participants with a recent history of adenoma were randomized within a 2x2 factorial design where they received 1 mg FA supplement or placebo, and aspirin (81 or 325 mg/d) [241]. This study was intended to investigate the possible preventive effect of FA on adenoma recurrence. All patients had undergone a complete colonoscopy with removal of all known polyps within three months of enrolment. Primary outcome was the recurrence of at least one adenoma, and secondary outcomes included the presence of advanced lesions, defined as tubulovillous, villous, or large adenomas with high-grade dysplasia, and multiplicity of new adenomas. There was no significant difference among the supplemented and placebo group at the first colonoscopy at three years (RR=1.04, 95% CI: 0.90-1.20, p=0.58) or the second at three to five years (RR=1.13, 95% CI: 0.93-1.37, p=0.23) in recurrence. However at the latter follow-up,

the FA group had a higher incidence of advanced lesions (RR=1.67, 95% CI: 1.00-2.80, p=0.05), and higher multiplicity (RR=2.32, 95% CI: 1.23- 4.35, p=0.02). The results suggest that FA does not reduce the risk of colorectal adenoma recurrence, but may promote transformation of preexisting preneoplasms [241]. Though all polyps were removed at enrolment, CRC has a long latency period and colonic mucosa can remain in a preneoplastic condition, sensitive to triggers promoting carcinogenesis. In addition, colonic folate concentrations are sensitive to rises in erythrocyte folate concentrations in individuals with polyps [243], thus it is a biologically plausible explanation that FA supplementation enhances the growth of established microscopic lesions. However, other clinical trials with three or more years of follow up suggest a null effect of FA supplementation on adenoma recurrence [238, 239].

A combined analysis of the Aspirin/Folate Polyp Prevention Study [241], Nurses' Health Study and Health Professional Follow-up Study [239], and the UK Colorectal Adenoma Prevention Study [238] was carried out by Figueiredo *et al* [244]. The combined analysis reported that FA supplementation was associated with a non-significant decrease in adenoma recurrence risk among subjects in the lowest category of baseline plasma folate (<11 nmol/L) and no effect of intervention among those in the highest category (>29 nmol/L) [244]. Though there was a higher occurrence of deaths among the placebo group, there was no significant benefit to FA supplementation after a follow-up period of up to 3.5 years, on the occurrence of new adenomas [244].

Intervention trials investigating FA alone or in combination of B vitamins and cancer risk are summarized in **Table 2.11**. These studies were designed to investigate the effect of FA supplementation with or without other B vitamins in the prevention of cardiovascular disease-related events, such as stroke, in CHD patients. FA supplementation lowered levels of

homocysteine, however FA alone, or in combination with other B vitamins, did not lower the risk of vascular outcomes, compared to controls receiving placebo [245-250].

A recent meta-analysis by Wien and colleagues [251] reports a borderline significant increase in the frequency of overall cancer in the FA supplemented group (RR=1.07, 95% CI: 1.00 to 1.14). Though a significantly higher incidence of CRC was not evident (RR=1.00, 95% CI: 0.83-1.21), prostate cancer incidence was significantly higher among those receiving FA supplements (RR=1.24, 95% CI: 1.03-1.49) [251]. Seven of the studies included in this publication use cancer incidence and/or mortality as a secondary endpoint; the first of which are events related to their primary health condition, such as preexisting cardiovascular disease or stroke. Interestingly, when dosage was taken into account, studies using 0.4 to 1.0 mg FA showed an increased risk in overall cancer risk, but those above 1.0 mg did not [251]. Of those studies which reported a null effect, the relatively short follow-up time may have attenuated the potential harmful effects of FA [244, 252]. To examine the effect of FA supplementation on cancer incidence and/or mortality as a secondary endpoint, a meta-analysis by Clarke *et al* included eight such randomized trials. These authors reported that FA yielded an average 25% reduction in homocysteine levels but had no significant on vascular outcomes including vascular mortality [253]. Furthermore, there was no significant effects of FA supplementation on overall cancer incidence (RR=1.05, 95% CI: 0.98-1.13) or cancer mortality (RR=1.00, 95% CI: 0.85-1.18) [253]. This finding has been supported by other, though smaller, meta-analyses [252, 254]. Despite the abundance of publications examining folate status and intake in the population, it is not clear whether higher levels of folate can directly increase the risk of developing adenomas or CRC. Biomarker studies of DNA methylation suggest that FA supplementation can increase genomic DNA methylation in patients with a history of adenomas or CRC, who may have

experienced a degree of hypomethylation caused by neoplastic transformation. In addition, studies with moderate levels of FA supplementation (0.5-1.0 mg/d FA) suggest a null or promoting effect on adenoma recurrence. Conclusions drawn from meta-analyses are conflicting and are limited by the heterogeneity of studies, particularly whether studies with cancer incidence or mortality as a secondary endpoint, were included or not, since these subjects have other preexisting conditions.

Despite the large number of epidemiological and clinical trials investigating folate status and CRC risk, the mechanisms remain unclear. Animal studies on the other hand, provide a clearer insight of the relationship between folate deficiency and supplementation on colorectal carcinogenesis.

Table 2.9. Summary of randomized control trials of FA supplementation as a chemopreventive agent in biomarkers of CRC*¶

Reference	Previous diagnoses (n)	FA dosage Duration	Endpoint	Outcome
Cravo *et al* [255]	CRC or adenoma (n=22)	10 mg/d 6 months	Rectal mucosa genomic DNA methylation	FA increased DNA methylation ($p<0.002$)
Cravo *et al* [256]	Chronic UC (n=25)	5 mg/d 6 months	DNA methylation	No change (p=N.S.)
Biasco *et al* [257]	Chronic UC (n=24)	15 mg/d 3 months	Rectal cell proliferation	FA reduced cell proliferation in the upper 40% of crypts ($p<0.01$)
Cravo *et al* [128]	Adenoma (n=20)	5 mg/d 3 months	Rectal mucosa genomic DNA methylation	FA increased DNA methylation in patients with single adenoma (p=0.05)
Kim *et al* [129]	Adenoma (n=20)	5 mg/d 1st: 6 months 2nd: 1 year	Rectal mucosa genomic DNA methylation P53 strand breaks	FA increased genomic DNA methylation at 1st and 2nd follow-up (p=0.001) FA decreased strand breaks at 1st and 2nd follow up ($p<0.02$)
Khosraviani *et al* [258]	Adenoma (n=11)	2 mg/d 3 months	Rectal mucosal cell proliferation	FA decreased cell proliferation
Nagothu *et al* [259]	Adenoma (n=20)	1 mg/d 1 year	LOH of *DCC*, *APC*, *TP53* DCC protein activity	FA protected DCC from LOH (p=N.S.) FA increased DCC expression ($p<0.02$)
Pufulete *et al* [237]	Adenoma (n=31)	400 μg/d 10 weeks	Rectal mucosal genomic DNA methylation	FA increased DNA methylation (p=0.09)

*****P value for trend; N.S.=non-significant. UC=ulcerative colitis, LOH=loss of heterozygosity, DCC=deleted in colon cancer, APC= adenomatous polyposis coli.

Table 2.10 Summary of randomized control trials of FA supplementation as a chemopreventive agent and adenoma recurrence *¶

Reference	Study/Subjects Previous diagnoses	Tx/Control	FA dosage Duration	Endpoint	RR (95% CI)	P-value
Paspatis *et al* [240]	Adenoma	31/29	1 mg/d FA 1^{st}: 1 year 2^{nd}: 2years	Recurrence	Recurrence in Tx vs. Ctrl: 1^{st}: 23% vs.38% 2^{nd}: 13% vs. 28%	 N.S. N.S.
Cole *et al* [241].	AFPPS Trial 21-80 years Adenoma	516/505	1 mg/d FA 1^{st}: 3 years 2^{nd}: 3-5 years	1^{o}: recurrence 2^{o}: advanced lesions	$1^{st}/1^{o}$: RR=1.04 (0.90-1.20) $1^{st}/2^{o}$:RR=1.32 (0.90-1.92) $2^{nd}/1^{o}$: RR=1.13 (0.93-1.37) $2^{nd}/2^{o}$:RR=1.67 (1.00-2.80)	N.S. N.S. N.S. P=0.05
Jaszewski *et al* [242]	Adenoma	49/45	5 mg/d FA 3 years	Multiplicity	OR=2.77 (0.06-0.84)	P=0.03
Logan *et al* [238].	ukCAP Trial <75 years Adenoma	320/307	0.5 mg/d FA 3 years	Recurrence	RR=1.07 (0.85-1.34)	N.S.
Wu *et al* [239].	HPFS, NHS Adenoma	338/334	1 mg/d FA 3-6.5 years	Recurrence	RR=0.82 (0.59-1.13) Low baseline folate: RR=0.61 (0.42-0.90) High baseline folate: RR=1.28 (0.82-1.99)	N.S. P=0.01 N.S.

*****P value for trend; N.S.=non-significant; RR=relative risk; HR=hazard ratio

¶ HPFS = Health Professionals' Follow-up Study, NHS = Nurses' Health Study, ukCAP = United Kingdom Colorectal Adenoma Prevention, AFPPS = Aspirin/Folate Polyp Prevention Study

Table 2.11. Summary of randomized control trials of FA supplementation and cancer incidence as a secondary endpoint *¶

Reference	Study/Subjects Previous diagnoses	Tx/Control	Intervention Duration	Endpoint	RR or HR (95% CI)	P-value
Zhu *et al* [260]	Atrophic gastritis	44/54	1st year: 20 mg FA 2nd year: 20 mg FA (twice weekly) +B12	Gastrointestinal cancer incidence	OR=0.12 (0.03-0.51)	P=0.004
Toole *et al* [249]	VISP Preexisting CVD	1827/1853	20 μg FA + B6+B12 2.5 mg FA +B6+B12 1.7 years	Cancer incidence	RR=0.98 (0.74-1.30)	N.S.
Lonn *et al* [248]	HOPE-2 Preexisting CVD	2758/2764	2.5 mg FA + B6+B12 5 years	Cancer incidence Cancer mortality	RR=1.06 (0.92-1.21) RR=0.99 (0.75-1.31)	N.S. N.S.
Jamison *et al* [261]	HOST Chronic renal disease	1032/1024	40 mg FA +B6+B12 3.2 years	Cancer incidence	RR=0.90 (0.65-1.24)	N.S.
Zhang *et al* [188].	WAFAC Study ≥42 years, women Preexisting CVD or ≥3 coronary risk factors	2721/2721	2.5 mg FA+B6+B12 7.3 years	Cancer incidence	HR=0.97 (0.79-1.18)	N.S.
Ebbing *et al* [262].	NORVIT, WENBIT Ischemic heart disease	3411/3426	0.8 mg FA+B12±B6 3.25 years	Cancer incidence Cancer mortality	HR=1.21 (1.03-1.41) HR=1.38 (1.07-1.79)	P=0.02 P=0.01
Armitage *et al* [250]	SEARCH Preexisting CVD	6033/6031	2.0 mg FA + B12 6.7 years	Cancer incidence Cancer mortality	RR=1.06 (0.96-1.17) RR=1.03 (0.87-1.22)	N.S. N.S.

*****P value for trend; N.S.=non-significant; RR=relative risk; HR=hazard ratio; OR=odds ratio

¶ CVD=cardiovascular disease, VISP=Vitamin Intervention for Stroke Prevention Trial, HOPE-2=Heart Outcomes Prevention Evaluation Study 2, HOST=HOmocysteinemia in kidney and end-stage renal disease Study, WAFAC=Women's Antioxidant and Folic Acid Cardiovascular Study, NORVIT=Norwegian Vitamin Trial, WENBIT=Western Norway B Vitamin Intervention Trial, SEARCH=Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine Trial.

2.4.2. Folate and its dual modulatory role in colorectal carcinogenesis

Folate provides essential substrates required for cell division and growth, one-carbon metabolism and biological methylation reactions. As such, inhibiting folate metabolism or preventing DNA replication in cancer cells inhibits tumor growth. Thus, evidence from epidemiological studies suggesting the role of folate as a cancer-preventive agent is contradictory. Where human studies are often confounded by the accuracy of retrospective food recall and limitations of biomarkers of folate status, animal studies investigating the relationship between folate and CRC have shown that the timing and dose of intervention is critical and important determinants of disease outcome (**Figure 2.13**). If FA supplementation is initiated prior to the establishment of preneoplastic lesions, such as ACFs, tumor development and subsequent progression is suppressed. However, if FA supplementation is initiated after preneoplastic lesions are present, FA can promote and facilitate progression to cancer [13]. Therefore, FA intervention plays a dual modulatory role where it can both suppress and promote colorectal carcinogenesis depending on the colonic environment, whether neoplastic transformation has occurred or not. The colonic mucosa is sensitive to manipulation of dietary and blood levels of folate, and we and others have effectively used rodent diets containing different levels of FA to modulate these levels [263-265].

2.4.2.1. Effects of folate deficiency and supplementation in the (pre)neoplastic colorectum

As mentioned previously, limiting folates in rapidly proliferating cancer cells causes tumor inhibition and is the main rationale behind the use of antifolate chemotherapy. This inhibitory mechanism is thought to be due to two reasons. First, limited availability of substrates for DNA synthesis can cause inefficient replication, thereby suppressing tumor growth. And second, limiting one-carbon units may reverse promoter hypermethylation of CpG islands

causing silencing transcription of tumor suppressor genes. The latter explanation has been shown in small rodent studies, but has yet to gain a firm approval from scientists. At the other end of the spectrum, in an environment where neoplastic transformation has already occurred, FA supplementation can primarily promote tumor growth by providing the substrates necessary for DNA replication at the accelerated rate characteristic of cancer cells. In addition, the excess FA can promote the de novo methylation of CpG islands of tumor suppressor genes and cause hypermutability of methylated cytosines in CG dinucleotide sequences. Lindzon *et al* show that carcinogenesis is dose-dependent and increasing doses of FA supplementation can promote neoplastic transformation [263]. In this model, ACFs were first established in all animals by azoxymethane (AOM), a DMH metabolite, prior to FA intervention, demonstrating FA supplementation promotes the transformation to neoplastic cells from preneoplastic cells.

2.4.2.2. Effects of folate deficiency and supplementation in the normal colorectum

The effect of FA intervention in normal colonic mucosa, prior to preneoplastic transformation has an opposite effect to that found in neoplastic colonic mucosa. FA supplementation in normal epithelia is thought to be cancer-preventive due to the sufficient supply of substrates for DNA synthesis, one-carbon metabolism and biological methylation reactions [13, 266]. Adequate folate can maintain DNA stability and integrity, provide sufficient substrate for optimal DNA repair, decrease mutagenesis and prevent aberrant DNA methylation. However, folate deficiency promotes preneoplastic transformation through several mechanisms [267, 268]. First, inadequate folate causes DNA and chromosome breaks due to uracil misincorporation [15, 269]. Folate deficiency, defined as <140 ng/mL erythrocyte folate or <3 ng/mL plasma folate, has been shown in human tissues to increase genomic instability by way of deficient methylation of dUMP to dTMP and incorporation of uracil into DNA by DNA

polymerase [15]. Second, limitation of substrates for cellular turnover can lead to impair DNA repair mechanisms and subsequently cause mutations which increasingly become detrimental to DNA integrity [117]. Lastly, folate deficiency may promote preneoplastic transformation by causing genomic DNA hypomethylation, permitting the transcription of genes which threaten the cellular integrity [117]. An early study by Cravo *et al* reported that in animals exposed to colorectal carcinogen, 1,2-dimethylhydrazine (DMH), animals fed folate-deficient diet from study initiation had a greater incidence of colonic neoplasia, compared to animals which received folate-sufficient and –supplemented diets prior to carcinogen exposure [267]. These results suggest that in an environment where preneoplastic transformation is induced, FA supplementation can be protective and prevent transformation.

The crucial difference between this study and the one by Cravo *et al* is the timing and dose of FA intervention. Where the former study [267] demonstrated that FA supplementation was chemopreventive prior to the establishment of preneoplastic lesions, the latter [263] demonstrated that FA supplementation was harmful after the establishment of ACFs, and in fact, promoted further neoplastic transformation. This relationship is elegantly demonstrated by Song *et al* in an $Apc^{+/-}Msh2^{-/-}$ mouse model [16]. These mice display an accelerated intestinal adenoma phenotype and develop numerous dysplastic colonic ACF, where the average time required for a tumor develop is estimated to be 27 days in the colon [270]. Therefore, animals received either 0 or 8 mg FA/kg diet at three or six weeks, corresponding to before and after the establishment of preneoplastic lesions, respectively. Among animals which began FA intervention at three weeks, FA supplementation significantly decreased the number of small intestinal adenomas by 2.7-fold ($p=0.004$), colonic ACFs by 2.8 fold ($p=0.028$) and colonic adenoma by 2.8-fold ($p=0.1$). In contrast, among animals which began FA intervention at six

weeks, the FA-deficient diet had a significantly reduced number of small intestinal adenomas by 4.2-fold (p=0.001), but had no effect on colonic ACFs and adenomas [16]. These studies, amongst others, elegantly suggest that folate deficiency and supplementation affect the colorectal mucosa differently and is dependent on timing and dose.

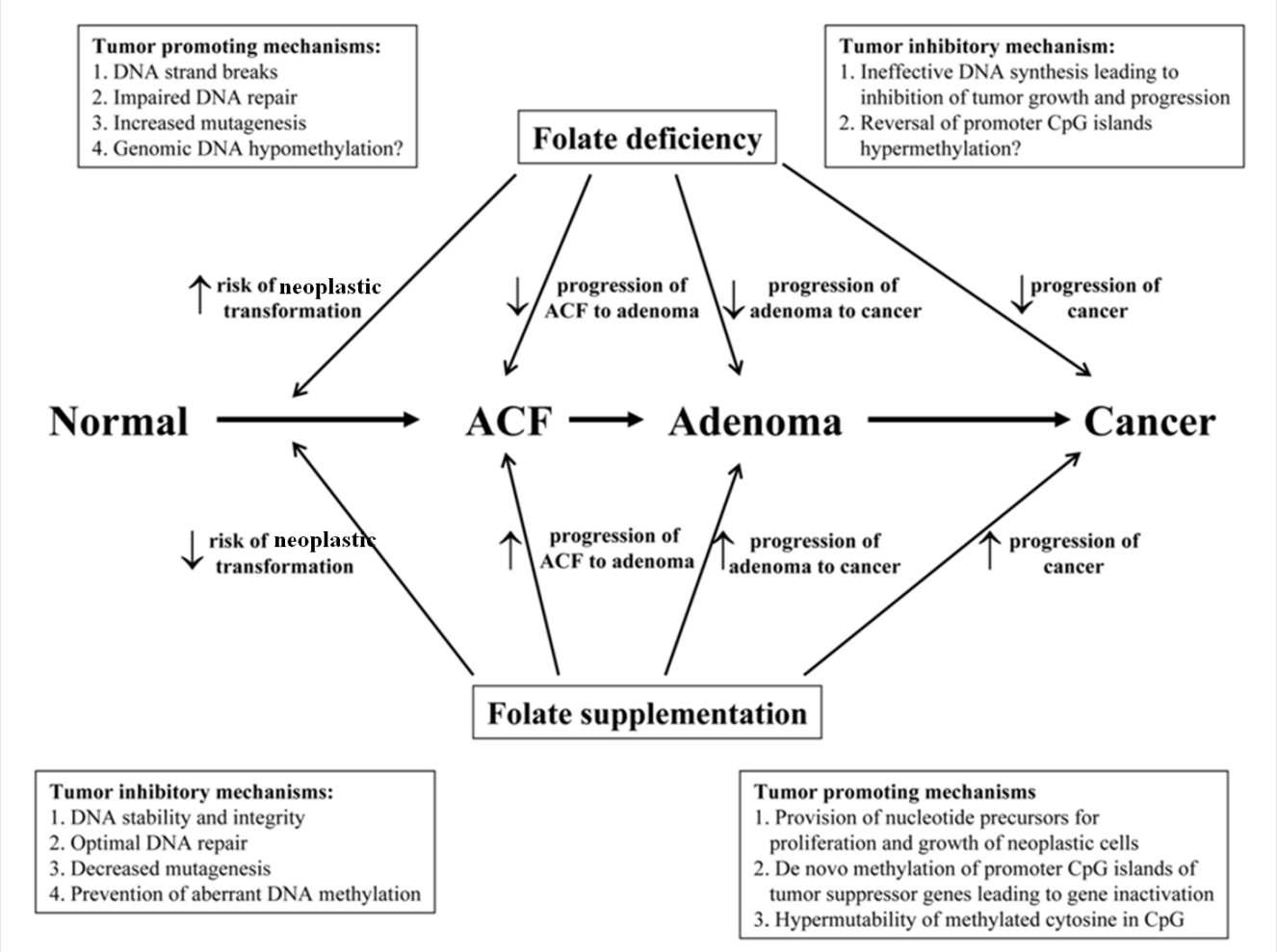

Figure 2.13. The dual modulatory role of folate deficiency and supplementation on colorectal carcinogenesis. Adapted and reprinted by permission from the publisher (John Wiley and Sons): [106].

Thus, at the population level, the increase in total folate intake can have very different outcomes depending on the age of the individual, since older individuals are more likely to harbor preneoplastic lesions [271]. Since fortification took place in the late 1990s and the surge

in supplement use came about in the early 2000s, those born in the late 2000s have been predicted to have a low risk of developing CRC. In contrast, those over the age of 50, a population likely harboring asymptomatic preneoplastic lesions, have been predicted to have a higher risk of developing CRC. In fact, Mason and colleagues elegantly illustrated the temporal association between fortification and the increase in CRC incidence [272] (**Figure 2.14**). Though there has also been an improved screening program in place, the increase in incidence cannot be attributed solely to earlier and improved detection [272]. This hypothesis is controversial and has been challenged [273], however given the role of folate and the carcinogenesis of CRC, it is a provocative hypothesis that warrants further studies to elucidate the relationship between FA and CRC.

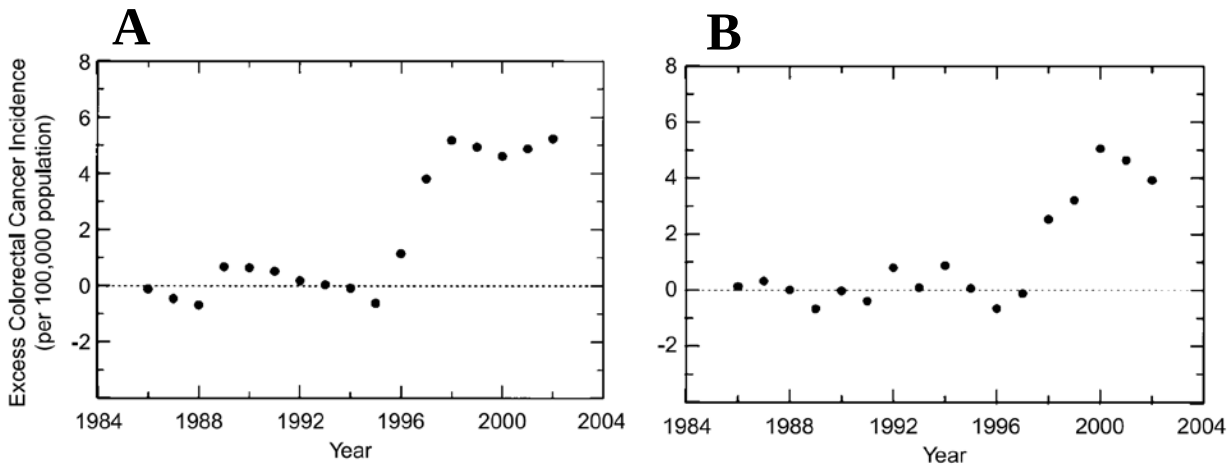

Figure 2.14 Excess of colorectal cancer incidence in the United States (A) and Canada (B). Incidence rate prior to FA fortification of the food supply was normalized as zero. In the postfortification period, CRC incidence has increased beyond the nonparametric 95% confidence intervals of prefortification trends or any increases accountable due to changes in screening programs. Given the biological mechanisms of folate in colorectal carcinogenesis, authors suggest it is biologically plausible that FA fortification is responsible for the increase in incidence rates. Adapted and reprinted with permission from the publisher (American Association for Cancer Research): [272].

Table 2.12. Summary of animal studies of FA supplementation and adenoma or colorectal cancer incidence.*¶

Reference	N; animal model	Dietary folate (mg FA/kg diet)	Duration	Endpoint	Outcome (in ascending order of groups, respectively) ±	P-value
Cravo *et al* [267]	Sprague Dawley rats DMH injection	Group 1: 8 Group 2: 0 Group 3: 8 + DMH Group 4: 0 + DMH	25 weeks	Incidence of colonic neoplasia (%) Animals with dysplasia (%) Animals with carcinoma (%)	0, 0, 43, 86 N/A, N/A, 28, 86[a] N/A, N/A, 14, 86[a]	N.S. P<0.05 P<0.01
Shivapurkar *et al* [274]	N=50 Fischer-344 rats AOM injection	Group 1:High fat, low fibre Group 2: High fat, low fibre + 3 mg	18 weeks	Animals with ACFs (%) Colon tumor incidence (%) Tumor multiplicity	52.6, 44.2 70, 80 1.3,1.4	P=N.S. P=N.S. P=N.S.
Kim *et al* [268]	N=40 Sprague Dawley rats DMH injection	Group 1: 0 Group 2: 2 Group 3: 8 Group 4: 40	15 weeks	Tumor incidence: - Microscopic (%) - Macroscopic (%)	 90, 90, 80, 70 70[a], 40[b], 10[c], 50[ab]	 P=N.S. P<0.03
Reddy *et al* [275]	N=72 Fischer-344 rats AOM injections	Group 1:control Group 2:2000	50 weeks	Tumor incidence Tumor size Tumor multiplicity	N/A Group 1 > Group 2 Group 1 > Group 2	P=N.S. P<0.05 P<0.05
Wargovich *et al* [276]	N=20 Fischer-344 rats AOM injections	Group 1: 0 Group 2: 2.5 Group 3: 5	2 weeks	Mean # ACFs	237, 181[a], 227	P<0.05
Song *et al* [16]	N=14 *Apc*$^{+/-}$*Msh2*$^{-/-}$	Group 1: 0 Group 2: 8	3 weeks 6 weeks	Mean total of small intestinal adenomas Mean # of ACF Mean # of colonic adenoma Mean total of small intestinal adenomas Mean # of ACF Mean # of colonic adenoma	299.4[a], 110.3[b] 55.1[a], 19.4[b] 1.7[a], 0.6[a] 70.0[a], 295.3[b] 34.5[a], 42.4[a] 2.4[a], 2.4[a]	P=0.004 P=0.028 P=N.S. P=0.001 P=N.S P=N.S.
Song *et al* [277]	N=79 *Apc*$^{-/-}$	Group 1: 0 Group 2: 2 Group 3: 8 Group 4: 20	3 weeks 6 weeks	Mean # small intestinal adenomas Mean # of colonic ACF Mean # colonic adenoma Mean # small intestinal adenomas Mean # of colonic ACF Mean # colonic adenoma	24.4, 23.3, 22.0, 16.9 1.30[a], 0.27[b], 0.20[b], 0.00[b] 4.60, 4.18, 3.2, 0.70 17.9, 26.3, 23.4, 17.9 0.33, 0.27, 0.56, 0.38 2.56, 2.82, 2.00, 4.43	P=N.S. P<0.05 P=N.S. P=N.S. P=N.S. P=N.S.
Carrier *et al* [278]	N=122 IL-2^{null}xβ_2m^{null} mice	Group 1: 0 Group 2: 2 Group 3: 8	32 weeks	Incidence of colonic lesions by type (%) - low-grade dysplasia - high-grade dysplasia/carcinoma *in situ*, invasive adenocarcinoma	 23.3[a], 9.3[b], 20.6[a] 33.3[a], 65.1[b], 35.3[a]	 P<0.05 P<0.05
Lindzon *et al* [263]	N=152 Sprague Dawley rats AOM injection	Group 1: 0 Group 2: 2 Group 3: 5 Group 4: 8	34 weeks	Mean # of ACF Mean # of crypts/focus Mean # of tumors/animal Mean # adenocarcinoma/animal Mean sum tumor diameter/animal (cm)	84.6[a], 93.4[ab], 108.1[ab], 137.9[b] 3.4[a], 3.5[ab], 3.4[a], 3.9[b] 2.8, 5.2, 5.0, 5.7 1.2, 1.8, 1.9, 2.2 0.5[a], 1.2[ab], 1.3[bc], 1.6[c]	P=0.003 P=0.06 P=0.002 P=0.006 P=0.001
Al-Numair	N=54; Albino rats	Group 1: 2	8 weeks	Mean total # ACF	65.3[a], 57.2[b], 26.3[c]	P<0.001

et al [279]	AOM injection	Group 2: 8 Group 3: 40				
Sie *et al* [265].	N=180; Sprague Dawley rats AOM injection	Maternal diet, pup diet Group 1: 2,2 Group 2: 2,5 Group 3: 5,2 Group 4: 5,5	31 weeks post weaning	Tumor incidence (%) Mean tumor multiplicity: - adenocarcinoma only - adenocarcinoma + adenoma Mean sum tumor diameter (cm): - adenocarcinoma only - adenocarcinoma + adenoma	75.6,85.7,60[a], 59.5[b] 1.67[a], 2.33[b], 2.04[a], 1.55[a] 1.73[a], 2.64[b], 2.04[a], 1.60[a] 0.76[a], 1.31[b], 0.97[a], 0.72[a] 0.78[a], 1.35[b], 0.98[a], 0.73[a]	P<0.05 P<0.05 P<0.05 P<0.05 P<0.05
Ciappio *et al* [280]	N=96; Apc^{1638N} mice	Maternal diet, pup diet Group 1: 0.5, 2 Group 2: 2, 2 Group 3: 8, 2	29 weeks post weaning	Animals with tumors (%) Invasive tumors (%)	55.6[a], 59.4[a], 20.8[b] 53.9[a], 17.7[b], 0	P<0.05 P<0.03
Kim *et al* [281]	N=84; C57BL/6 mice Young (4 months) Old (18 months)	Group 1: 0 Group 2: 2 Group 3: 8	20 weeks	Uracil content in colonic DNA	Young: 0.19[a], 0.19[a], 0.59[b] Old: 0.26, 0.18, 0.23	P<0.05 P=N.S.

*****P value for inverse trend; N.S.=non-significant.

¶ DMH=1,2-dimethylhydrazine, AOM=azoxymethane

±Different superscripted letters indicate a statistically significant difference between groups.

2.4.3. Folate and its role in chemosensitivity to 5-fluorouracil chemotherapy

Very little is known about optimal intracellular folate concentrations, which simultaneously minimize effects of toxicity and also preserves the effectiveness of treatment. Antifolate-based chemotherapy agents are structurally similar to folate, and compete with folate for cellular uptake, retention and export. Approved antifolate treatments and the effects of folate or FA supplementation are summarized in **Table 2.13**. Other agents such edatrexate, lometrexol, ralitrexed, and nolatrexed are still considered investigational, but preliminary *in vitro* and *in vivo* studies suggest that modest FA supplementation can decrease toxicity whilst preserving the effects of treatment.

Table. 2.13. Approved antifolate cancer treatments and the effects of folate or FA supplementation.

Agent	Target enzyme(s)	Approved uses	Effect of folate/FA supplementation
Methotrexate (MTX)	DHFR (1^{o}), TS, MTHFR, GART, AICARFT	Leukemia Breast cancer Lymphoma (Burkitt's and non-Hodgkin's) Osteosarcoma Head and neck cancer	*In vitro*: FA increased drug sensitivity in lymphocytic leukemia cells [282] *In vivo*: FA increased survival time in L1210 lymphoid leukemia mice receiving a dosage 1 hour before or with MTX [283] Clinical: No difference in blood MTX levels receiving FA supplements [284]
Pemetrexed	TS (1^{o}), DHFR, GART	Malignant pleural mesothelioma (MPM) Non-small-cell lung cancer	*In vitro*: Folinic acid reversed drug effects in CCRF-CEM leukemia cells [285]; FA was less effective than folinic acid in protecting cells [286] *In vivo*: FA decreased toxicity without affecting drug effects [287] Clinical: Gastric patients receiving FA responded to treatment [288]; MPM patients receiving FA supplements had less toxicity, tolerated more cycles and a greater survival [289]

Several groups provide evidence that it is the increased levels of intracellular folate which enhance treatment [18, 290, 291]. This is generally understood to be due to the expanded pool of 5,10-methyleneTHF which further stabilizes the inhibition of the ternary complex. An animal study by Branda *et al* suggest that FA supplementation can enhance chemotherapeutic

efficacy [292]. Xenograft models of breast cancer were placed on a low, regular, or high FA diet in conjunction with cyclophosphamide, 5FU or doxorubicin. Animals receiving the high FA diet had consistently greater tumor inhibition than the low FA group. Furthermore, the high FA diet supplemented with daily 50 mg/kg FA intraperitoneal (IP) injections had a significantly higher survival percentage than those on the FA-replete (2 mg FA/kg diet) or low FA diet (0 mg FA/kg diet) when administered 75 mg/kg 5FU ($p=0.025$), suggesting that mammary tumors were more responsive to 5FU in a folate-rich environment, in a dose-responsive manner [292]. Furthermore, FA-deficient animals were significantly more sensitive to 5FU-induced toxicity compared to the FA-replete group, and additional FA supplementation was further protective [292]. Similar findings have been shown in other animal studies [18]. A follow-up study however found that in rats treated with bolus 5FU (single IP doses from 110-546 mg/kg), the FA-injected rats (2 mg/kg FA plus daily 50 mg/kg FA IP injection) had severe anemia, increasing renal damage and leukopenia compared to those on a cereal, FA-deficient or FA-replete diet (2 mg FA/kg diet) indicative of treatment-related toxicity [293]. By contrast to the previous study, this study used very high concentrations of 5FU administered in single doses, which may have suppressed the effects of FA. Therefore FA supplementation has shown to enhance sensitivity to 5FU when doses are moderately low. In addition, it is unknown whether there is a threshold effect of FA supplementation or 5FU dosage, where at certain levels for either FA or 5FU, the favorable interaction no longer is evident.

There is very limited data on response to chemotherapy in relation to pretreatment folate status in colorectal patients. Clinical data suggest that folate status does not predict patient outcome and remains relatively stable throughout the course of chemotherapy [294] and a large observational study in the United States confirm that higher levels of prediagnostic plasma folate

were not associated with overall CRC mortality, and there were no harmful effects in individuals in the highest quintile of plasma folate levels [295].

Studies investigating the MTHFR C677T polymorphism and sensitivity to 5FU generally suggest a better prognosis among individuals bearing this mutation [207]. Accumulating preclinical and clinical studies provide evidence that an expanded pool of 5,10-methyleneTHF is required for optimal TS inhibition [290, 296-300]. Since the 677TT genotype results in a thermolabile protein leading to an intracellular accumulation of 5,10-methyleneTHF, theoretically, tumors with this mutation are more sensitive to 5FU, compared to wild-type tumors. In a xenograft model using nude mice, Sohn *et al* demonstrate a 78% inhibition of xenograft growth in human colon cancer cells expressing the 677TT mutation compared to those expressing the wild-type MTHFR (36% inhibition) [301]. Similarly, in a clinical setting, CRC patients with unresectable liver metastases with 677CC, 677CT, and 677TT genotypes, demonstrated 5FU response rates of 40%, 21% and 56% in, respectively [302], although, better response did not translate to a survival advantage. A similar finding was observed among 43 patients with metastatic CRC [303]. This study showed that carriers of the 677T allele were more likely to respond to 5FU treatment compared to non-carriers [303]. However, this relationship remains controversial. An *in vitro* study of nineteen human cell lines suggested that the MTHFR C677T mutation had no effect on 5FU sensitivity, and in fact the MTHFR A1298C mutation had a larger effect on chemosensitivity [304]. Altogether, MTHFR appears to play an important role in intracellular homeostasis and shuttling of 5,10-methyleneTHF for nucleotide biosynthesis or biological methylation reactions, and may potentially have an impact on sensitivity to 5FU.

2.4.4. Mechanisms of folate-induced drug resistance

Though there has been some evidence regarding the relationship between the modulation of intracellular folates and chemosensitivity to 5FU, it is a relatively novel and provocative concept that higher levels of folate may induce drug resistance, at levels beyond what is achieved by modulation of MTHFR genotypes. There are three proposed mechanisms in which this may occur: increased TS expression, competitive inhibition of FPGS, and the induction of MDR.

2.4.4.1. Increased TS expression

In the presence of 5FU metabolites, the proportion of free, or unbound, TS decreases as FdUMP binds to TS and forms the inhibitory ternary complex [79]. Unbound TS inhibits its own transcription by blocking transcription factors from binding to its gene, effectively preventing further expression [305, 306]. Higher TS expression has been associated with decreased sensitivity to 5FU and poorer prognosis of CRC [307]. Since the main mechanism of 5FU is the inhibition of TS, concentration of TS beyond the suppressive capacity of FdUMP would continue to contribute to thymidylate synthesis. A polymorphism in the 5' untranslated region of the *TS* promoter can affect chemosensitivity to 5FU, where those bearing the homozygous mutation 3R/3R, resulting in higher expression of TS, has a significantly lower response to treatment, compared to the 2R/2R and 2R/3R genotypes [17]. Therefore, TS expression serves as a marker for 5FU efficacy, and lower concentrations of the enzyme are associated with a favorable response. However, in environments of high folate, unbound TS can dissociate from its gene and is no longer able to suppress its own transcription resulting in a increased transcription and thus a higher level of TS [306]. In fact, mathematical modeling has demonstrated that with increasing folate, thymidylate synthesis is the sole folate-dependent mechanism that continues to increase [308] (**Figure 2.15**). The other mechanisms, purine

synthesis and the rate of biological methylation, demonstrate trends of deceleration [308]. It is well established that alterations in TS expression, by way of gene amplification, leads to resistance to TS-inhibitors, whether innate [304, 309, 310] or acquired [311, 312]. Considering higher TS expression levels are associated with a poorer prognosis [307], it is worth investigating whether higher folate intake can impact response to chemotherapy. Sohn *et al* reported that, although there was no difference in TS protein expression, TS catalytic activity was higher in colon cancer cells expressing the MTHFR 677TT genotype [301]. Therefore, consistent with the changes in intracellular folate concentrations compared to wildtype cells, the 677TT mutation cells increase TS activity without affecting its protein expression.

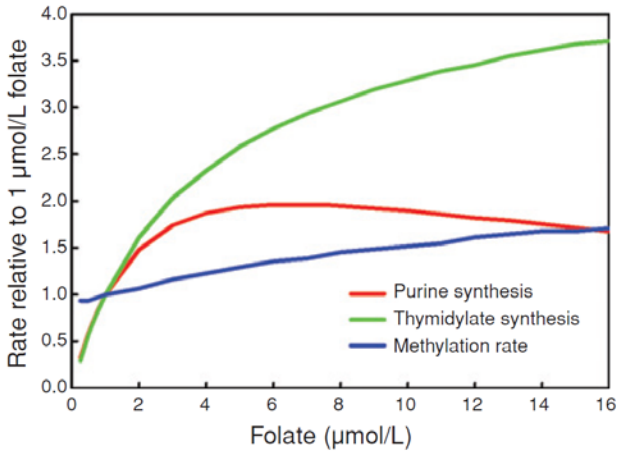

Figure 2.15. Purine and thymidylate synthesis and methylation rate with increasing intracellular folate levels. A concentration of 1 µmol/L was chosen as the reference value for the reason that human colonic cells averaged 1 µmol/L, with a range of 0.2-6.9 µmol/L. Adapted and reprinted by permission from the publisher (American Association for Cancer Research): [308].

2.4.4.2. Competitive inhibition of FPGS

Since expanded of pools of 5,10-methyleneTHF enhances 5FU efficacy, Sohn *et al* demonstrated *in vitro* that compared with cells expressing endogenous FPGS, when FPGS was overexpressed, cells had significantly higher concentrations of long chain folate polyglutamates

and enhanced chemosensitivity to 5FU [313]. Some antifolate drugs such as MTX require intracellular polyglutamylation by FPGS. However in the presence of higher folates, antifolate drugs must compete for cellular sequestration [314] and subsequent treatment efficacy. In leukemia cells, Mauritz *et al* demonstrated that resistance to antifolate drugs result at the level of FPGS, due to competition with natural folates [314]. The study further suggests that the highest levels of resistance was observed against drugs that are most dependent on poyglutamylation for their pharmacologic activity [314]. These findings are further supported by Zhao *et al* who demonstrated in L1210 leukemia cells, a reduction in antifolate drug sensitivity with increasing concentrations of folate [315].

2.4.4.4. Induction of MRPs

Recent *in vitro* studies postulate that high levels of intracellular folate can increase the activity of some membrane transporters responsible for the efflux of folates, antifolates and other anti-metabolite [19]. MRP1 through MRP5 and BCRP have shown to modulate intracellular concentrations of folates by increasing efflux activity [19]. Briefly, MRP1 and MRP3 are not viable in folate-depleted environments, but at physiological concentrations, total folate and antifolate content is significantly decreased [96, 97, 99, 316]. MRP2 binds antifolates such as MTX for efflux, however the relationship with intracellular folates are not clear [97, 317]. Hooijberg *et al* [96] suggest that 10-formylTHF is readily effluxed in ovarian cells in MRP1, MRP2 and MRP3-overexpressing cells. Vesicular membrane transport of FA, LV and antifolates by MRP4 and MRP5, and to a lesser degree BCRP, have been reported [98, 318], but whether their activity is influenced by intracellular folate concentrations, is unknown. Ifergan *et al* reported a markedly reduced activity of BCRP in folate-deprived environments [104]. In addition to *in vitro* (reviewed in [319]) and *in vivo* evidence [18], a higher folate status among cancer

patients appear to attenuate efficacy and toxicity of some antifolates [287, 320]. Hooijberg *et al* [319] suggest an inverse U-association with respect to the therapeutic efficacy of antifolates, where at low folate levels patients are affected by toxicity, and at high folate levels there are is an increased activity of MDR efflux pumps (**Figure 2.16**). Thus, given that colonic folate concentrations are sensitive to total folate intake [321], increased folate concentrations from intake and supplements may induce drug efflux and thereby decrease chemosensitivity in CRC treatment.

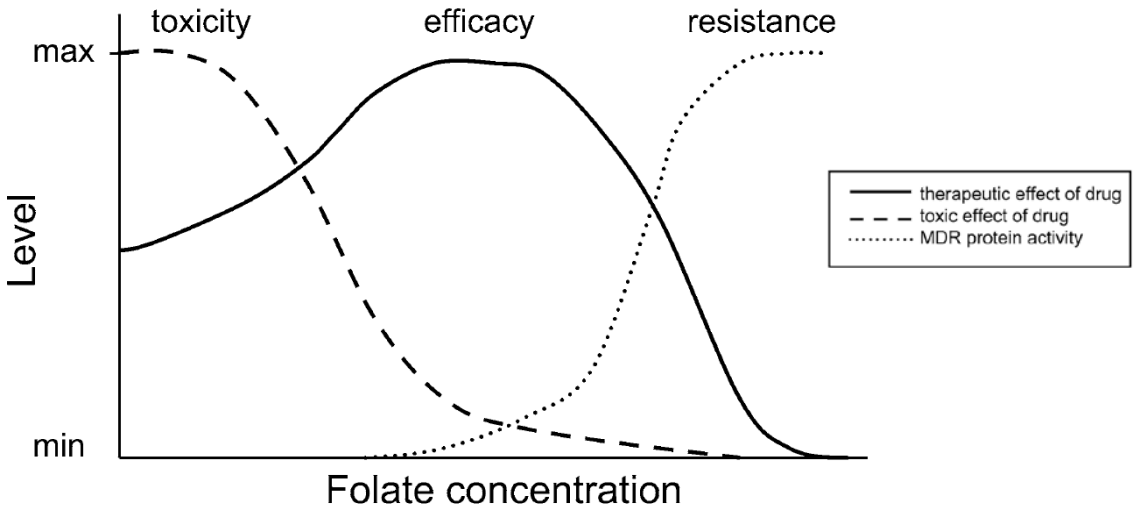

Figure 2.16. A hypothesized model of the relationship between intracellular folate concentration and chemotherapeutic efficacy. A low levels, there are treatment-related toxicities which supercede the effectiveness of the drug. At high levels, folate however can induce mechanisms of resistance. Adapted and reprinted by permission from the publisher (Springer): [319].

2.5. Animal models

In vivo models of CRC are extensively used since given the complexity of the disease, and it is often challenging, and unethical, to investigate hypotheses in the human population. Clinical studies involving environmental factors, such as dietary assessment, are challenging in nature to monitor, recall retrospectively, and infer relationships to specific disease outcomes. Experimental diets for laboratory animals using varying levels of FA in amino acid-defined diets effectively modulate folate levels, as confirmed by concentrations in plasma and liver, indicative of short and long term folate status, respectively [263-265, 322].

DHFR expression in rodents is up to 35-fold greater than what is found in human liver and mucosa [323], implicating whether results from rodent studies can be extrapolated to reflect the pharmacokinetics of folates in humans. In addition, the density of FR in rodent kidney is not analogous to humans, such that rodent kidney excretes a greater concentration of folate in urine, compared to humans, which reabsorbs and secretes folates with bile [323]. Although there are obvious specie differences of absorption, metabolism and excretion of nutrients and drugs in rodents compared to humans, animal models are an invaluable tool in experimental designs, refinement of current regimens and the development of new ones. Established genetic or chemical carcinogen rodent models of CRC provide an opportunity to investigate new interventions and drugs in a controlled experimental model to assess metabolism, dosage and duration, toxicity and effect on disease state and overall health.

Administering 5FU-based chemotherapy following induction of (pre)neoplasms by carcinogen is an effective method of investigating the effects of treatment while controlling for the timing of intervention [324-327]. Sakamoto *et al* used a DMH-induced rodent model to define the relationship between *de novo* and salvage pathways for pyrimidine synthesis in

response to 5FU-based chemotherapy [325]. Kuwa *et al* investigated the effects of long term administration of a 5FU derivative (UFT, uracil-tegafur) plus leucovorin on colorectal tumors induced with DMH in rats. Induction of CRC by carcinogen is a proven and effective rodent model of human relevance, in intention-to treat studies.

Xenograft models are useful in the study of potential therapeutics and novel treatment drugs. Studies using xenograft models possess a particular advantage to observe treatment response for several reasons. First, strains are immunocompromised, lacking an essential component of immune function such as T-cells, or lacking the entire thymus gland, thereby allowing the effects of intervention alone to be observed without the innate immune response confounding the results. Second, cell lines of known genotypic characteristics can be selected based on research needs. For example, commonly used human colon carcinoma cell lines HCT116 and HT29 cells differ in MSI and p53 status, where the former is MSI-positive with wildtype p53 function, and the latter exhibits opposite characteristics [328]. Finally, xenografts provide an opportunity to observe the effects of intervention and in an *in vivo* model, which can differ greatly from cell culture where growth conditions are simulated and often difficult to interpret in humans. Commonly, cells are injected subcutaneously because xenografts are easily visible, allowing measurements to be carried out using a simple caliper since immunocompromised rodents lack hair. Recently, orthotopic transplantation have been asserted as a preferable model over subcutaneous tumor development in xenograft models of cancer [329]. However, imaging systems like magnetic resonance or computed tomography are necessary since colon tumors are not macroscopically visible [330]. By contrast, mammary tumors can be orthopically implanted since rodent mammary glands can be palpated for tumors and easily measured with calipers without the aid of imaging systems.

CHAPTER 3: PROBLEM FORMULATION AND RESEARCH OBJECTIVES

3.1. Current state of problem

As an important mediator of nucleotide synthesis and one-carbon metabolism, folate is an essential nutrient, where deficiency in this vitamin can result in megaloblastic anemia, NTDs, and other adverse birth outcomes. Supplementation of FA, the synthetic and more bioavailable form of the vitamin, can correct anemia and low white blood cell count induced by inadequate folate. In addition, maternal FA supplementation has proven to be an effective and safe method of preventing NTDs in the fetus. As such, mandatory FA fortification of staple foods, such as breads and breakfast cereals, has been implemented in over fifty countries in the past two decades[331]. As a result of this public health initiative, the prevalence of NTDs decreased by approximately 50% in Canada [2]. Furthermore, erythrocyte folate concentrations have also dramatically increased among those who are not women of child-bearing age or at risk of NTDs [332]. In fact, a recent study suggests that folate deficiency (erythrocyte folate <305 nmol/L) is virtually nonexistent in Canada [165]. FA fortification was intended to provide an additional 100-200 μg/day and decrease the prevalence of preventable birth defects, and thus, can be considered a public health success.

In addition, the growing popularity of dietary supplement use has drastically increased erythrocyte folate levels such that supplement use has become the greatest indicator of folate status [7, 166]. Despite the lack of evidence supporting the benefits, greater than 50% of the U.S. and Canadian population report regularly using at least one multivitamin or single-nutrient supplement daily [4, 5].

However, there is a growing body of evidence that high folate status may be associated with adverse health outcomes. High FA supplementation has been implicated in masking vitamin

B12 deficiency [151], accelerated cognitive decline in individuals with low B12 status [152, 177], impaired natural killer cell cytotoxicity [176], resistance to certain drugs [151], and cancer [106]. Tumor promoting effects of FA have been implicated in cancers of the prostate [184], breast [187, 188], pancreas [185, 186], and colorectum (Tables 1.5 to 1.8), to name a few.

The application of folate as a potential chemopreventive agent suggest that the relationship between folate and carcinogenesis is not as straightforward as previously thought. Folate is an essential cofactor in the *de novo* synthesis of nucleotides and thus is the main contributor to DNA synthesis, replication and maintenance of genomic integrity [13]. In normal cells, folate deficiency can initiate neoplastic transformation, due to the insufficient supply of nucleotides for normal DNA replication to occur, whereas supplementation ensures sufficient provision of substrates, thereby reducing the risk of neoplastic transformation [13]. In contrast, once preneoplastic lesions have been established in the colorectum, FA supplementation can promote the progression of these lesions and accelerate transformation by providing nucleotide substrates to rapidly dividing cells [13]. Limiting folate in a (pre)neoplastic environment causes ineffective DNA synthesis, resulting in inhibition of tumor growth, and as such, is the rationale for antifolate- and antimetabolite-based chemotherapy [13].

The popular belief that dietary supplement use can ward off chronic diseases, such as cancer, has fueled the ubiquitous habit among cancer patients and survivors to adopt this new practice [333]. In particular, newly diagnosed individuals are most receptive and responsive to activities perceived to be health-promoting [9, 11, 174]. Though improvement of dietary and exercise behaviours may be recommended by their physicians, undertaking other interventions such as dietary supplement use is often an autonomous decision and is not discussed [9]. Up to 81% of cancer survivors have reported using a daily average of two supplements [11, 169-171].

Though supplement use is highest among breast cancer patients, greater than 50% of CRC patients currently take supplements during the course of their treatment [172, 173, 334]. This is of most utmost concern since 5FU is the main cornerstone of all CRC chemotherapy, which is based on the inhibition of TS, a critical enzyme involved in folate metabolism. Despite the little to no scientific evidence supporting any anticancer effects of supplements and achieving folate levels beyond what is deemed safe, FA may interact and interfere with 5FU-based chemotherapy. Furthermore, there is recent evidence that high FA levels may affect chemosensitivity by inducing mechanisms of drug resistance [19, 96]. To date, there are no *in vivo* studies which have investigated whether FA supplementation can affect response to 5FU.

3.2. Research rationale

Erythrocyte folate levels have increased dramatically due to mandatory FA fortification and supplement use [2, 162]. In fact, over 50% of U.S. and Canadian adults regularly use dietary supplements, potential benefits of which are largely unknown [4, 5]. Commonly, supplements contain a minimum of 400 μg FA, the current RDA; however individuals take upwards to 5 mg FA. This is of particular concern among CRC patients who are on 5FU-based chemotherapy, mechanisms of which are based on the interruption of folate metabolism. Observations from i*n vitro* studies show that the overexpression of MRPs (MRP1, MRP2 and MRP 3) demonstrate a 32-38% decrease in total folate content and effectively modulate folate homeostasis [96]. Further characterizations of MRPs have demonstrated that transporters able to bind antifolates are sensitive to intracellular folate concentrations, and effectively decrease antifolate drug efficacy by pumping out both antifolates and folates[319]. Specific to 5FU, MRP5 and MRP8 have been identified to efflux nucleotide analogues, such as FdUMP and FUTP [90, 92]. Recently, MRP5 has been shown to transport folates and also mediate resistance against antifolates [91].

Furthermore, a growing body of evidence suggest that folate has a dual modulatory role in colorectal carcinogenesis, FA supplementation may promote the progression of existing (pre)neoplastic lesions [13]. This presents an immediate concern among cancer patients who harbor established neoplasms and their habitual supplement use could potentially interfere with the efficacy of their treatment. To our knowledge, there have been no studies investigating whether FA supplementation at levels achieved by diet and regular supplement use could affect chemosensitivity to 5FU via folate-induced mechanisms of resistance.

3.3. Research hypothesis

We hypothesize that FA supplementation may interfere with 5FU-based chemotherapy and decrease its efficacy by increasing the activity of efflux transporters binding metabolites of 5FU, effectively conferring resistance.

3.4. Research objective

The objective of this study is to explore whether supplemental levels of FA can i) affect tumor growth indicative of chemosensitivity, and if so then ii) explore whether folate-induced drug efflux is responsible, in an well-established xenograft model of human colon carcinoma.

CHAPTER 4: THE EFFECT OF FOLIC ACID SUPPLEMENTATION ON CHEMOSENSITIVITY TO 5-FLUOROURACIL IN A XENOGRAFT MODEL OF HUMAN COLON CARCINOMA

4.1. Introduction

Folates provide essential substrates for the *de novo* synthesis of nucleotides and biological methylation reactions. In the past decade, the habitual use of FA supplements has increased significantly in addition to fortification of our food supply, and as a result erythrocyte folate levels have dramatically increased [4-6, 162]. Preclinical animal studies have shown that FA supplementation can promote the progression of preneoplastic lesions [13, 106]. This is of utmost concern among CRC patients receiving 5FU chemotherapy. Given that greater than 50% of cancer patients regularly use FA-containing supplements throughout their treatment [173], this is a concern for a large proportion of the population. Furthermore, recent research suggest that higher intracellular folate can increase the efflux activity of anticancer agents by MDR transporters, inducing resistance to 5FU, effectively decreasing the efficacy of treatment [319]. Thus, this study investigated whether expanded intracellular folate pools provided by FA supplementation could affect chemosensitivity of colon cancer cells to 5FU chemotherapy and affect activity of MRP1, MRP5 and MRP8.

4.2. Specific objectives

To determine whether 8 and 25 mg FA can i) modulate xenograft growth in a xenograft model of human colon carcinoma, compared to mice ingesting 2 mg FA (control), and ii) to explore whether the changes in chemosensitivity to 5FU can be attributed to increased drug efflux by membrane transporters sensitive to intracellular folate and 5FU concentrations (MRP1, MRP5 and MRP8).

4.3. Materials and methods

4.3.1. Animal model

An immune-compromised, or nude, mouse model was used in this study. These models lack an intact thymus and cannot generate T-cells, due to a mutation in the *FOXN1* gene, thus being an ideal recipient for many types of tissue and tumor grafts without rejection [335]. Athymic nude mice from Harlan Laboratories were originally thought to be Balb/c congenic, but were later reported by the National Cancer Institute to be outbred. Compared to its inbred cousins, these animals can exhibit heterogeneity in response to interventions and treatments.

4.3.2. Experimental design

Male athymic nude mice (Hsd: Athymic nude-Foxn1^{nu}) at four weeks of age, were purchased from Harlan Laboratories (Madison, WI, USA) (**Figure 4.1**). Animals were acclimated for one week during which they received free access to water and amino acid-defined diet (Dyets, Bethlehem, PA, USA) containing 2 mg FA/kg [336]. After one week, they were injected subcutaneously with 1.0x10^6 HCT116 human colon carcinoma cells in each flank. Two to three weeks later, animals with established xenografts measuring 60-100 mm^3 were randomized into one of three diet groups, 2, 8 or 25 mg FA/kg diet. Within these groups, animals were further randomized to receive an IP injection of either saline vehicle (0.9% NaCl, 100 µL) or 5FU (20 mg/kg body weight, 100 µL) + leucovorin (1 mg/kg body weight, 100 µL) for five consecutive days. Xenograft dimensions were measured by a digital micrometer caliper three times a week, and volumes were calculated as V= ½ (LW^2), where W is defined as the largest measurement, and L is defined perpendicular to W [337, 338]. All animals were housed in sterile polycarbonate microinsolators (1-3 per cage) at a temperature (24 ± 2^{o}C) and humidity (50%) controlled, negative pressure environment, with a 12-hour light/dark cycle. Cages, bedding and

environmental enrichment were changed on a biweekly basis, in which all materials were autoclaved-sterile. The use of wire-bottomed stainless steel cages to prevent coprophagy was not used due to ethical considerations. Animal care and use were in accordance with the Canadian Council on Animal Care 1984 guidelines. This animal protocol was approved by the Animal Care Committee at the University of Toronto, Toronto, ON (Protocol 20008638).

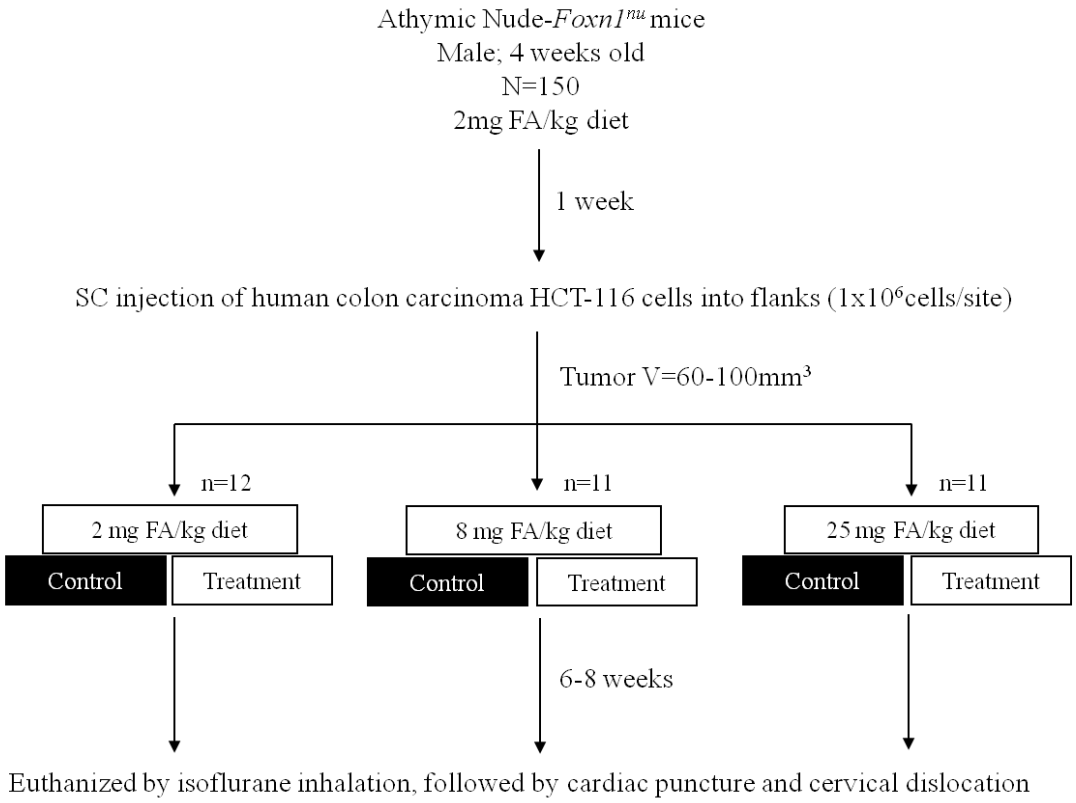

Figure 4.1. Study design. Athymic nude mice were injected with HCT116 human colon carcinoma, subcutaneously in each flank. Once xenografts reached 60-100 mm^3, they were randomized into 2, 8 or 25 mg FA/kg diets. Within each diet group, they were further randomized to saline or treatment (20 mg/kg 5-fluorouracil + 1 mg/kg leucovorin) by intraperitoneal injection for five consecutive days. Xenografts were measured three times a week by digital micrometer caliper, and animals were euthanized 6-8 weeks following randomization to treatment groups.

4.3.3. Diets

Gamma-irradiated sterile diets in pellet form were used (Dyets, Bethlehem, PA, USA). These L-amino acid defined diets provide a method of modulating dietary folate levels in rodents in a highly predictable manner and have been extensively used in previous studies from our laboratory [263-265, 277, 278]. Casein-based diets were avoided for the reason that they contain high levels of folate and cannot induce folate deficient or supplemental states in a predictable manner. Three different diets differing only in FA levels were used (**Tables 4.1 to 4.3**). The control diet (2 mg FA/kg diet) supplies the basal dietary requirement (BDR) of folate for rodents [339, 340] and is representative of the RDA of 400 µg for humans. This level was determined by taking into consideration that the experimental diets contain approximately 4000 kcal/kg diet, and therefore in 2000 kcal, approximately 0.5-1 mg FA would be ingested. Since the estimated daily caloric intake for humans is 2000 kcal, this amount of FA expressed relative to caloric content, is similar to the RDA for humans. Furthermore, the 2 mg FA/kg diet meets the growing rodent's requirement for nutrients, as indicated by their hematological profile and folacin levels in whole blood and liver [336]. The diet containing 8 mg FA/kg diet provides four times the BDR, and represents a daily intake of 1.6 mg/day in humans, to represent a total folate intake from fortified foods and regular supplement use of 1.0 mg FA. Finally, the diet containing 25 mg FA/kg diet provides 12.5 times the BDR, and represents a daily intake of 5 mg/day in humans. Such high levels of FA intake are taken by pregnant women and patients with certain medical conditions receiving antifolate treatment. Both diets and water were provided *ad libitum* and changed weekly, where remaining pellets were discarded. Diets were stored long-term in -20^{o}C, and let thawed in 4^{o}C overnight for short-term use.

4.3.4. Cell culture

Human colon carcinoma HCT116 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in standard RPMI-1640 medium (Invitrogen, Gaithersburg, MD, USA). Growth medium was supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin, and 0.1% fungizone. Cells were maintained at 37^{o}C in 95% humidity and 5% CO_2 and passaged every three days.

Table 4.1. Experimental L-amino acid defined diets nutrient composition

Ingredient (g/kg diet)	2 mg FA # 517774	8 mg FA # 517904	25 mg FA # 517905
L-Alanine	3.5	3.5	3.5
L-Arginine (free base)	11.2	11.2	11.2
L-Asparagine	6	6	6
L-Aspartic acid	3.5	3.5	3.5
L-Cysteine	3.5	3.5	3.5
L-Glutamic acid	35.0	35.0	35.0
Glycine	23.3	23.3	23.3
L-Histidine (free base)	3.3	3.3	3.3
L-Isoleucine	8.2	8.2	8.2
L-Leucine	11.1	11.1	11.1
L-Lysine HCl	14.4	14.4	14.4
L-Methionine	8.2	8.2	8.2
L-Phenylalanine	11.6	11.6	11.6
L-Proline	3.5	3.5	3.5
L-Serine	3.5	3.5	3.5
L-Threonine	8.2	8.2	8.2
L-Tryptophan	1.74	1.74	1.74
L-Tyrosine	3.5	3.5	3.5
L-Valine	8.2	8.2	8.2
Total L-amino acid	**171.44**	**171.44**	**171.44**
Dextrin	407	407	407
Sucrose	193	193	193
Cellulose	50	50	50
Corn oil (Stab. 0.015% BHT)	100	100	100
Salt mix #21006	57.96	57.96	57.96
Vitamin mix #317756	10	10	10
Choline chloride	2	2	2
Sodium bicarbonate	6.6	6.6	6.6
Folic acid/sucrose premix (1 mg/g diet)	**2**	**8**	**25**

Table 4.2. Composition of mineral and salt mix in experimental diets (Salt mix #21006)

Ingredient (g/kg diet)	
Calcium carbonate	14.6000
Calcium phosphate, dibasic	0.17000
Sodium chloride	12.37000
Potassium phosphate, dibasic	17.16000
Magnesium sulfate, anhydrous	2.45000
Manganese sulfate, monohydrate	0.18000
Ferric citrate	0.62000
Zinc carbonate	0.05400
Cupric carbonate	0.05400
Potassium iodide	0.00058
Sodium selenite	0.00058
Chromium postassium sulfate	0.01900
Sodium fluoride	0.00230
Molybdic acid, ammonium salt	0.00120
Sucrose	10.27534

Table 4.3. Composition of vitamin mix in experimental diets (vitamin mix #317756)

Ingredients (g/kg diet)	
Thiamin HCl	0.006
Riboflavin	0.006
Pyridoxine HCl	0.007
Nicotinic acid	0.030
Calcium pantothenate	0.016
Cyanocobalamin	0.00005
Vitamin A palmitate (500 000 IU/g)	0.008
Vitamin D_3 (400 000 IU/g)	0.0025
Vitamin E acetate (500 IU/g)	0.100
Menadioine sodium bisulfate	0.00080
Biotin	0.00002
Sucrose	9.82363

4.3.5. Xenograft establishment in nude mice

Confluent dishes were washed three times with phosphate buffered saline (PBS) (Invitrogen, Gaithersburg, MD, USA) and trypsinized for 10 minutes in 37^{o}C. Cells were suspended in serum-free media and diluted to 5x10^{6} cells/mL, and 200 µL of cell suspension was immediately administered by subcutaneous injection into each flank of each mouse by a hired technician. The flank was chosen preferentially over the shoulder for ease of handling, as well as

a superior take rate and less hindrance of mobility. Xenografts were established within three to four weeks post injection, and randomized to one of six groups (**Figure 4.1**).

4.3.6. Drug preparation and administration

LV (Sigma, F7878, powder) was dissolved in saline with 2 M NaOH, to a concentration of 1 mg/kg body weight of each rodent. Similarly, 5FU (Sigma, F6627, powder) was dissolved in saline with 2M NaOH, to a concentration of 20 mg/kg body weight [301, 341]. All drugs were prepared daily within 30 minutes of injection and kept on ice until injection. One hundred µL LV was administered one hour prior to 100 µL 5FU, both by IP injection. Animals receiving treatment were handled as a chemical hazard in a full-exhaust biosafety cabinet, due to the respiratory hazards associated with exposure to 5FU. Animals receiving saline vehicle were administered IP 100 µL saline at the same time points.

4.3.7. Sample collection

At necropsy, animals were anesthetized with isoflurane inhalation and subsequently killed by cardiac puncture, using a pre-heparinized needle, and cervical dislocation. Cardiac blood was kept on ice until harvesting of organs was complete. Blood was then taken off the ice, and let stand at room temperature for 30 minutes and centrifuged for 10 minutes at 2500 rpm. Plasma was aliquoted and stored at -80^{o}C for future analysis of folate and homocysteine concentrations. Five percent by volume of 1% ascorbic acid (Sigma, A5960, crystalline) was added to the former aliquot to prevent folate oxidation [342]. Liver was snap frozen in liquid nitrogen and stored at -80^{o}C. The left lateral hepatic lobe was identified and set aside for folate concentration determination, for the reason that different lobes have shown to metabolize different nutrients at varying concentrations and affinities [343]. Xenografts were harvested, one half fixed in 10% neutral-buffered formalin for histological and immunohistochemical analyses,

and one half was snap frozen for DNA and RNA extraction. Macroscopic swelling of inguinal lymph nodes were identified by their reddish-brown colour indicating response to infection, inflammation or cancer cells [344]. Normal lymph nodes are recognized by their clear-yellow colour. Macroscopically swollen inguinal lymph nodes were fixed in formalin for histological analysis.

4.3.8. Determinations of plasma, liver and xenograft folate concentrations

Plasma, liver and xenograft folate concentrations were determined using a standard microbiological *Lactobacillus casei* microtiter plate method [345], following treatment with chicken pancreas conjugase for liver and xenograft. *L. casei* grows proportionally to increasing folate concentrations, thereby its turbidity as measured by spectophotometry, is indicative of sample folate concentrations.

4.3.8.1. Folic acid standard preparation

Ten milligrams FA was dissolved in 10 mL of ddH_2O with 5 µL 10 M NaOH to 1 mg FA/mL. pH was adjusted to pH 7 to 8 with HCl and the concentration was verified by spectrophotometry (280 nm). The solution was diluted to 2 ng/mL with 0.1 M KPO_4 buffer (1.05 g KH_2PO_4, 0.4 g K_2HPO_4, 0.1 g Na ascorbate, 100 mL ddH_2O, filter sterilized), aliquoted, and stored at -80^{o}C.

4.3.8.2. Lactobacillus casei stock preparation

Two hundred microliters *L. casei* ATCC 7469 stock was incubated with Lactobacillus MRS broth for 18 hours at 37^{o}C. Under aseptic conditions, cells were centrifuged and the supernatant was decanted. Cell pellet was resuspended in 180 mL of MRS broth and 20 mL of cold 100% glycerol. Solution was aliquoted and stored at -80^{o}C.

4.3.8.3. Chicken pancreas conjugase preparation

Chicken pancreas acetone powder (Difco, 0459-12-12) was dissolved in 0.1 M KPO_4 buffer (**Table 4.4**) and incubated for 6 hours at 37^{o}C under a blanket of toluene. Toluene was removed and the solution was centrifuged at 10 000 x g for 15 minutes. The supernatant was collected and added to an equal volume of tricalcium phosphate (BioRad Get HTP was rehydrated: 1 part HTP: to 6 parts 0.1 M KPO_4 buffer, per 10g HTP). The solution was stirred for 30 minutes at 4^{o}C and centrifuged at 10 000 x g for 30 minutes at 4^{o}C. The supernatant was cooled to 4^{o}C, added to an equal volume of 95% ethanol, and left overnight at -20^{o}C. The day after, the solution was centrifuged at 10 000 x g for 30 minutes, supernatant was removed, and resuspended in 50 mL of cold 1.0 M KPO_4 buffer. Ten grams of Dowex-1 (BioRad AG1-X8) was added, stirred for 1 hour at 4^{o}C, and filtered at 4^{o}C. The solution was aliquoted and stored at -80^{o}C.

4.3.8.4. Liver and xenograft tissue preparation for folate concentration determination

Liver and xenograft tissue were weighed and extracted with extraction buffer at 10 and 4 times the organ weight, respectively. Extraction buffer was composed of 1 g Na ascorbic acid, 1 g Bis-Tris (Sigma), 50 mL ddH_2O, 35µL β-mercaptoethanol. Samples were boiled in water bath for 15 minutes, cooled on ice for 15 minutes, and homogenized. Samples were centrifuged at 5000 rpm at 4^{o}C for 20 minutes, supernatant was collected, and stored at -80^{o}C. For folate concentration determination, 0.1 M KPO_4 buffer and chicken pancreas conjugase was added to liver and xenograft samples in 15:1:4 and 4:1:5 ratios, respectively. Samples were vortexed and incubated for 2 hours at 37^{o}C. Liver samples were diluted further with 0.1 M KPO_4 buffer in 1:9 ratio.

4.3.8.5. Plasma, liver and xenograft folate concentration determination

Three microlitres *L. casei* stock was inoculated in 3 mL Lactobacillus MRS broth and incubated in a shaker at 37^{o}C for 16-18 hours. Five hundred microlitres of culture was reinoculated in 2.5 mL Lactobacillus MRS broth and further incubated in a shaker at 37^{o}C for 6.5 hours. Bacterial growth, or turbidity, was confirmed by optical density as measured by spectrophotometer at 650 nm (O.D. of 1.8 is considered optimal).

One hundred and fifty microlitres of 0.1 M KPO_4 buffer was added to each well in a clear 96-well microtiter plate. An equal volume of folic acid standard was added to specified wells, and serially diluted for an eight-point standard curve. Both solutions were filter sterilized and made fresh on the day of the assay. Similarly, samples (plasma with ascorbic acid, 5 µL; diluted liver extract, 10 µL; xenograft extract, 2.5 µL) were added to specified wells. To the same wells, 0.1 M KPO_4 buffer was added to bring up the total well volume to 300 µL. These wells were serially diluted three times for four measurements per sample.

Table 4.4. Composition of 0.1 M KPO_4 buffer and folic acid media for folate assay

0.1 M KPO_4 buffer	9.4 g folic acid media
1.05 g KH_2PO_4	0.05 g Na ascorbate
0.4 g K_2HPO_4	ddH_2O (up to 100 mL)
0.1 g Na ascorbate	
ddH_2O (up to 100 mL)	Boil for 2 minutes to dissolve and cool.

Under aseptic conditions, *L. casei* inoculum was centrifuged at 5000 rpm for 5 minutes to sediment bacteria. Supernatant was decanted and bacteria was resuspended in 3 mL sterile folic acid medium (**Table 4.4**), and centrifuged at 5000 rpm for 5 minutes – this was repeated two more times. After the final resuspension, the inoculum mixture was diluted with folic acid media (24x), and further diluted (40x). One hundred fifty microlitres of the 40x diluted inoculums was added to each well of the plate, to bring the final volume to 300 µL. Plates were covered with

mylar sealers and incubated at 37^{o}C for 16-17.5 hours. Plates were read by spectrophotometry at 650 nm, using SoftMax software.

4.3.9. Determination of plasma homocysteine concentration

The Axis ® Homocysteine EIA kit was used to determine homocysteine concentration (Abbott Laboratories, Mississauga, ON, Canada). The assay releases bound homocysteine and enzymatically converts free homocysteine to SAH. All reagents included in the kit were equilibrated to room temperature overnight prior to the assay. The sample pre-treatment solution was made no more than one hour prior to the start of the assay. This solution consisted of assay buffer (phosphate buffer, sodium azide), adenosine/DTT (adenosine, dithiothreitol, citric acid), and SAH-hydrolase (recombinant SAH hydrolase, trisbuffer, glycerol, methylparaben) at specified volumes, 45 mL, 2.5 mL and 2.5 mL, respectively. Subsequently, 12.5 µL of calibrators, samples and controls were combined, diluted with 250 µL of pre-treatment solution and mixed well. Microcentrifuge tubes containing sample solution were incubated at 37^{o}C for 30 minutes. Prior to cooling, 250 µL enzyme inhibitor (merthiolate, phosphate buffer) was added, mixed, and incubated for 15 minutes at room temperature. Then, 250 µL adenosine deaminase (adenosine deaminase, phosphate buffer, sodium azide, BSA, phenol-red dye) was added, mixed, and incubated for 5 minutes at room temperature. Twenty-five microlitres of sample solution and 200 µL anti-SAH antibody (monoclonal mouse-anti-SAH antibody, BSA, merthiolate) were added to each well of a SAH-coated microtitre plate, and incubated at room temperature for 30 minutes. The plate was washed four times with 350 µL of 1:9 diluted wash buffer (phosphate buffer, merthiolate, Tween 20, BSA) and wells were emptied on paper towel. To the plate, 100 µL enzyme conjugate (rabbit anti-mouse antibody enzyme conjugate, BSA, horse radish peroxidase, blue dye) was added to each well and incubated for 20 minutes at room temperature.

Wells were washed with 1:9 diluted wash buffer and emptied on paper towel, as previously described. To the plate, 100 µL substrate solution (N-methyl-2-pyrrolidon, propyleneglycol) was added to each well and incubated at room temperature for 10 minutes. Lastly, 100 µL stop solution (0.8 M sulphuric acid) was added to each well and placed on an automatic plate shaker to ensure even distribution. Within 15 minutes, the microtitre plate was read at 450 nm by spectrometry using Softmax software to measure peroxidase activity. Absorbance is inversely related to the concentration of homocysteine in the sample.

4.3.10. Quantitative reverse transcriptase-PCR

4.3.10.1. Total RNA extraction

To isolate RNA from selected xenograft samples, the RNeasy Microarray Tissue Mini Kit was used (Qiagen, catalogue no. 73304). In brief, this kit integrates phenol/guanidine-based lysis and silica-membrane purification to extract RNA from tissue samples. Snap-frozen xenograft specimens were homogenized in 1.0 mL QIAzol lysis reagent (Qiagen, catalogue no. 79306). Lysates were incubated at room temperature for 5 minutes to allow the dissociation of nucleoprotein complexes. To the samples, 200 µL chloroform was added, manually shaken, let incubate at room temperature for 3 minutes, and centrifuged at maximum speed for 15 minutes at 4^{o}C. The upper, aqueous phase was collected and combined with an equal volume of 70% ethanol. Sample solutions were added to an RNeasy Mini spin column, centrifuged briefly at room temperature, and eluate was discarded. In order to efficiently remove all DNA, on-column DNA digestion was performed using an RNase-free DNase set (Qiagen, catalogue no. 79254). To the spin column, 350 µL Buffer RW1 was added, centrifuged briefly at room temperature, and eluate was discarded. Next to the spin column, 10 µL DNase I stock solution and 70 µL Buffer RDD was added, mixed gently by inverting tube and incubated for 15 minutes at room

temperature. Then, to the spin column, 350 µL Buffer RW1 was added, briefly centrifuged at room temperature, and eluate was discarded. To wash the membrane, 500 µL Buffer RPE was added directly to the spin column, centrifuged briefly at room temperature, and eluate was discarded, twice. Finally, to elute RNA, the spin column was placed in a new tube, 30 µL RNase-free water was added, and centrifuged for 1 minute at room temperature, twice. Purity of extracted RNA was determined by spectrophotometry, measuring the ratio of absorbance (A_{260}/A_{280}). Samples were diluted in Tris-EDTA buffer, pH neutral in a 1:8 ratio and readings of $A_{260}/A_{280} > 2$ were achieved. An absorbance ratio greater than 2 indicates pure RNA. Extracted RNA was aliquoted and stored at -80^{o}C until use.

4.3.10.2. Synthesis of cDNA

cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen, catalogue no. 205311). In brief, this kit combines the effective removal of genomic DNA, enzymatic reverse transcription from RNA and the subsequent denaturation of active enzymes. Components of the kit were thawed, centrifuged and kept on ice. Two microlitres gDNA wipeout buffer (7x), 2 µL RNase-free water and 10 µL template RNA (150ng/mL) was combined in a 0.6mL microcentrifuge tube, incubated at 42^{o}C for 2 minutes, and immediately placed on ice. Reverse-transcription master mix was prepared as follows: 4 µL Quantiscript RT Buffer (5x), 1 µL RT Primer Mix, 1 µL Quantiscript Reverse Transcriptase and 14 µL denatured template RNA. Samples were incubated at 42^{o}C for 30 minutes then at 95^{o}C for 5 minutes to inactivate reverse transcriptases. Synthesized cDNA was immediately placed on ice and stored at -20^{o}C until use.

4.3.10.3. Quantitative RT-PCR

Quantitative RT-PCR was performed using the ViiA7™ Real-Time PCR System (Applied Biosystems, Life Technologies) according to the manufacturer's instructions. This

system was chosen because of its robust and precise qRT-PCR results for a variety of genomic research applications. Reactions were performed in a 10 µL volume with 0.06-0.3 µL 10-50 µM primers, 4.9 µL Fast SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies), RNase-free water and 0.6 µL cDNA template. The primer sequences in the reaction mixtures are listed in **Table 4.5**. Cycling conditions were as follows: 2 minutes at 50^{o}C, then 10 minutes at 95^{o}C, followed by 40 cycles with 15 second denaturation at 95^{o}C, 1 minute primer annealing at 60^{o}C, 15 seconds of fragment elongation at 95^{o}C. Samples were run in triplicate on each plate, and repeated at least twice on separate days.

Table 4.5. Primer sequences of housekeeping and investigative genes used in quantitative RT-PCR.

Gene	Primer Sequence
Housekeeping genes	
GAPDH [346]	Sense: 5'-ACCACAGTCCATGCCATCAC-3' Antisense: 5'-TCCACCACCCTGTTGCTGTA-3'
β-actin [347]	Sense: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' Antisense: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'
Investigative genes	
TS [346]	Sense: 5'-CCAAACGTGTGTTCTGGAAGG-3' Antisense: 5'-GCCTCCACTGGAAGCCATAA-3'
DHFR [346]	Sense: 5'-ACCTGGTTCTCCATTCCTGAG-3' Antisense: 5'-CCTTGTGGAGGTTCCTTGAGT-3'
MRP1 [93]	Sense: 5'-ATGTCACGTGGAATACCAGC-3' Antisense: 5'-GAAGACTGAACTCCCTTCCT-3'
MRP5 [102]	Sense: 5'-CGAAGGGTTGTGTGGATCTT-3' Antisense: 5'- GTTTCACCATGAAGGCTGGT-3'
MRP8 [347]	Sense: 5'-GAAGTCCTCCTTGGGCATGGC-3' Antisense: 5'-TTATCTCAGTGAAGAAGTGGCTGT-3'

4.3.11. Histological evaluation of tumors

Two pathologists (Dr. Cathy Streutker and Dr. Andrea Grin, St. Michael's Hospital), who were blinded to diet and treatment assignments, evaluated the H & E prepared tumor slides. Tumors were evaluated using a three-tier grading scheme where low, medium and high grades

were defined as well, moderate and poorly differentiated adenocarcinoma cells, respectively. The degree of necrosis was evaluated on a three-tier scale where specimens exhibited less than 33%, 33-66% or greater than 66%. Mitotic counts were carried out to evaluate proliferation of tumor sections per 10 high-power fields (HPF).

4.3.12. Ki-67 staining

Paraffin slides were cut and stained by core lab at St. Michael's Hospital. Briefly, samples embedded in paraffin were first deparaffinized in water. A process for antigen removal was performed with citrate buffer pH 6.0 and steamed for 20 minutes, the blocked with normal serum for 30 minutes. Primary antibody Ki-67 (1:200) was incubated for 1 hour and ImmPRESS anti-rabbit IgG was incubated for 30 minutes. Diamino benzidine chromogen was applied to the samples for 3 minutes, followed by counter-staining with hematoxylin for 30 seconds. Samples were washed with PBS between each step. Slides were then dehydrated with xylene and mounted. Whole-slides were scanned at the Advanced Optical Microscopy Facility at the University Health Network (Toronto, ON). Images were viewed with Aperio ImageScope Version 10.0 at 20x magnification. Areas of 408 033 μm^2 were chosen as representative captures of the whole slide. Staining was detected by setting hue value, hue width and colour saturation threshold to 0.1, 0.4, and 0.05, respectively. Positive staining was quantified by setting Iwp(high), Iwp(low)=Ip(high), Ip(low)=Isp(high) and Isp(low) to 150, 150, 0 and 80, respectively. Positivity scores were calculated as a ratio of total positive to total staining (positive and negative).

4.3.13. Statistical Analyses

A sample size of 150 animals, 25 per group, was required to be 80% certain of detecting a difference of 0.65 standard deviations in xenograft growth between any level of diet. This level

was chosen based on previous studies from our lab demonstrating this effect size, and the spread of data of tumor growth (unpublished data).

The study design was based on two factors, diet and treatment, and thus a two-way analysis of variance (ANOVA) with the inclusion of an interaction term was used to analyze the following parameters: body weight, initial xenograft volume, folate and homocysteine concentrations, measures of xenograft burden (mean sum and volume), and epithelial proliferation. Prior to testing, dependent variables were tested for normality with a histogram. As well, Levene's test of equality of error variances was not significant, thus meeting the assumptions required to utilize the ANOVA model for statistical testing. If a significant interaction (alpha level $p \leq 0.05$) was observed, Tukey's post-hoc test was performed. Metastases to local lymph nodes is a binary outcome, where metastasis was macroscopically evident or not, at the time of necropsy. For this variable, a binary logistic regression was used. Xenograft growth is a continuous variable with increasing scatter in data as the study progressed, violating the assumptions of an ANOVA model. In this case, a generalized estimating equation analysis was used. This statistical testing is appropriate for xenograft growth because it assumes that measurements are correlated with one another. Relative tumor volume (RTV) was chosen as the unit of measure of raw tumor volume to control for any differences in initial tumor volume. To calculate RTV, each tumor volume was divided by its individual initial tumor volume. Independent parameters, such as age and body weight, were included in the regression model as predictor variables if the coefficient of determination increased with its addition. An autoregressive correlation was used because it assumes that measurements taken closer in time from the same animal are more strongly correlated than measures taken further apart in time.

Percentages of positivity scores of Ki-67 staining per group were determined by classifying samples into three groups by positivity scores, and dividing it by the total number of samples per group. The same samples were used to measure the degree of necrosis, and percentages of degrees of necrosis per group were similarly determined. Two-way ANOVA was used to determine whether there was any difference between groups using diet and treatment as fixed factors.

Relative gene expression was measured using the delta delta Ct method (ΔΔCt). This method was chosen over absolute quantification because we were interested in the changes in gene expression relative to each of the diet and treatment groups. First, Ct values across experiments were compared per sample, and outliers were removed to limit standard deviations to < 0.5. The ΔCt method requires an internal control to normalize the number of reactions for the amount of cDNA added to the reaction. Suitable housekeeping genes were chosen for internal controls by validation with end-point PCR. The ΔΔCt method in addition requires a calibrator, an untreated control. The gene expression profile of wildtype HCT116 propagated *in vitro* was used as the calibrator. Thus, the data using the ΔΔCt method presents the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated HCT116 cell culture. Gene expression data were analyzed using the ViiA7^{TM} Real-Time PCR System (Applied Biosystems, Life Technologies). Non-parametric Kruskal-Wallis test was used to compare gene expression differences between groups.

Statistical tests were performed using SPSS 20.0 for PC (IBM, Armonk, New York) and graphs were prepared in Microsoft Excel 2007 and SPSS Output. All statistical tests were two-sided and considered significant at alpha level $p \leq 0.05$.

4.4. Results

4.4.1. Sample size and growth curves

Thirty-four of 146 animals developed a xenograft at the site of injection. Of these, 88% of animals (30/34) had established xenografts unilaterally. Animals that did not harbor a xenograft by 16 weeks of injection were euthanized. Growth curves were similar among the animals prior to and after randomization, and there was no difference in body weight at the time of diet intervention.

4.4.2. Initial xenograft volume

Eleven to 12 animals were randomized to each of the diet groups as xenografts grew to the appropriate volume (**Table 4.6**). Volumes were not statistically different among the six groups ($p=0.67$).

Table 4.6. Baseline characteristics at the time of randomization.*

Diet	2 mg FA		8 mg FA		25 mg FA		*P* value*
Intervention	Control	Treatment	Control	Treatment	Control	Treatment	
Body weight (g)	29.2 ± 0.7	30.4 ± 1.2	28.7 ± 1.3	28.3 ± 1.5	28.0 ± 1.4	29.8 ± 0.9	0.23
Initial tumor V (mm^3)	103.3 ± 23.6	120.7 ± 15.6	136.0 ± 35.4	113.5 ± 21.3	91.4 ± 13.3	116.8 ± 16.9	0.67

Results are expressed as mean ± SEM. * denotes a significant main effect due to diet.

4.4.3. Folate and homocysteine concentrations

For each of the 34 animals, plasma and hepatic folate concentrations were analyzed (**Figure 4.2**). Plasma folate concentrations were statistically different among the three diet groups ($p=0.002$). Animals on the 2 mg FA diet had significantly lower folate concentrations compared to animals on the 8 or 25 mg FA diet ($p=0.043$ and $p=0.001$, respectively). However, the latter two diet groups were not statistically different from each other ($p=0.1$), suggesting a threshold effect. Treatment had no effect on plasma folate concentrations ($p=0.8$).

Hepatic folate concentrations were also statistically different among the three diet groups ($p=0.035$), but it was not dose-dependent (**Figure 4.3**). Animals on the 8 mg FA diet showed trends of higher folate concentrations compared to animals receiving the 2 and 25 mg FA diet ($p=0.076$ and $p=0.018$, respectively). Concentrations between the latter two groups were not statistically different between each other ($p=0.5$). Treatment had no effect on hepatic folate concentrations ($p=0.5$).

Folate concentrations were measured for all 41 xenografts. Concentrations were statistically different among the three diet groups in a dose-dependent manner ($p=0.002$) (**Figure 4.4**). Xenografts of the 2 mg FA diet had lower folate concentrations compared to those harvested from animals on the 8 and 25 mg FA diet ($p\leq0.05$ and $p<0.001$, respectively). The 25 mg FA group had higher folate concentrations than the 8 mg FA group, showed borderline significance ($p=0.05$). This dose-dependent trend reflects the unique ability of aggressive cancer cells to acquire folate necessary for their growth and proliferation. Treatment had no effect on xenograft folate concentrations ($p=0.4$).

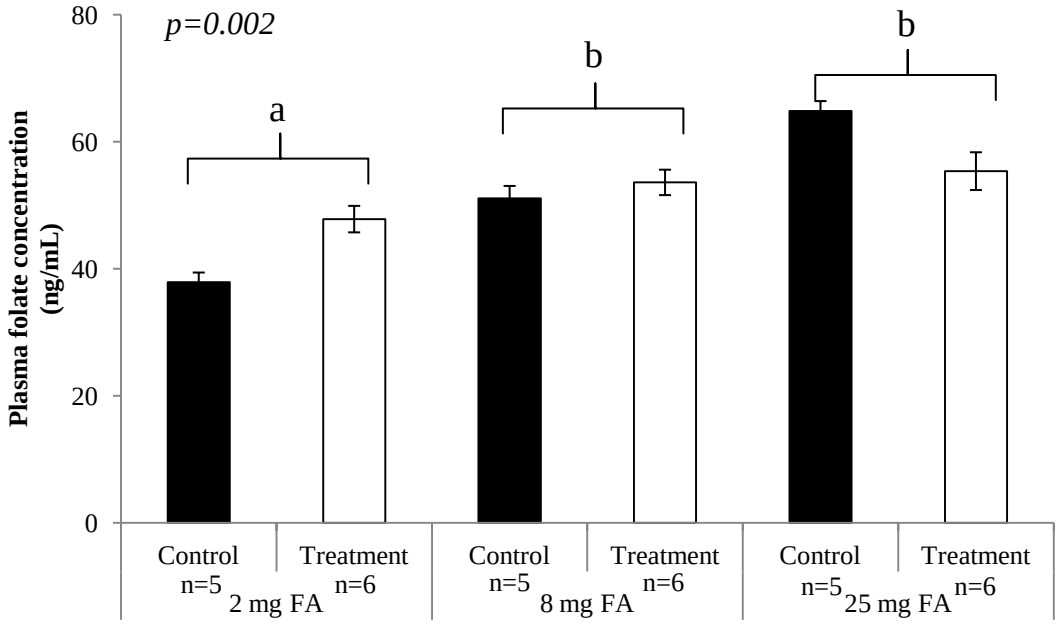

Figure 4.2. The effect of folic acid supplementation and 5-fluorouracil treatment on plasma folate concentrations. Results are expressed as mean ± SEM. P value denotes the main effect of diet. Means statistically different between diet groups are denoted with different letters ($p \leq 0.05$).

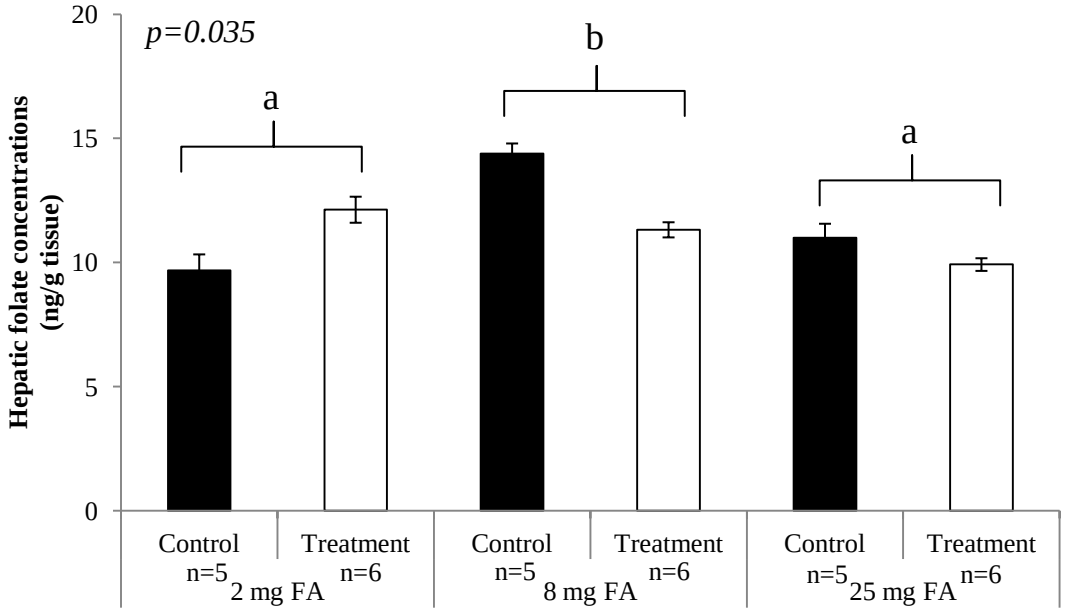

Figure 4.3. The effect of folic acid supplementation and 5-fluorouracil treatment on hepatic folate concentrations. Results are expressed as mean ± SEM. P value denotes the main effect of diet. Means statistically different between diet groups are denoted with different letters ($p \leq 0.05$).

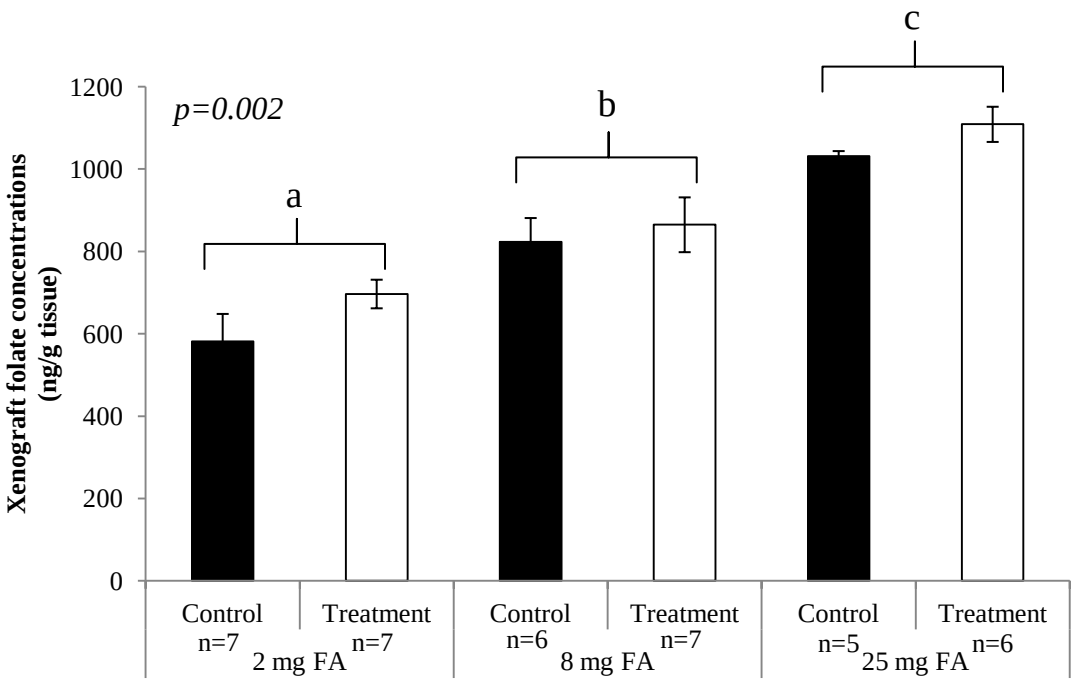

Figure 4.4. The effect of folic acid supplementation and 5-fluorouracil treatment on xenograft folate concentrations. Results are expressed as mean ± SEM. P value denotes the main effect of diet. Means statistically different between diet groups are denoted with different letters ($p \leq 0.05$).

Plasma homocysteine concentrations were not statistically different among the three dietary groups (p=0.1) (**Figure 4.5**). This is consistent with previous rodent studies suggesting that homocysteine is not lowered by FA supplementation beyond what is already achieved by the BDR, 2 mg FA [268, 348]. Treatment had no effect on homocysteine concentrations (p=0.4).

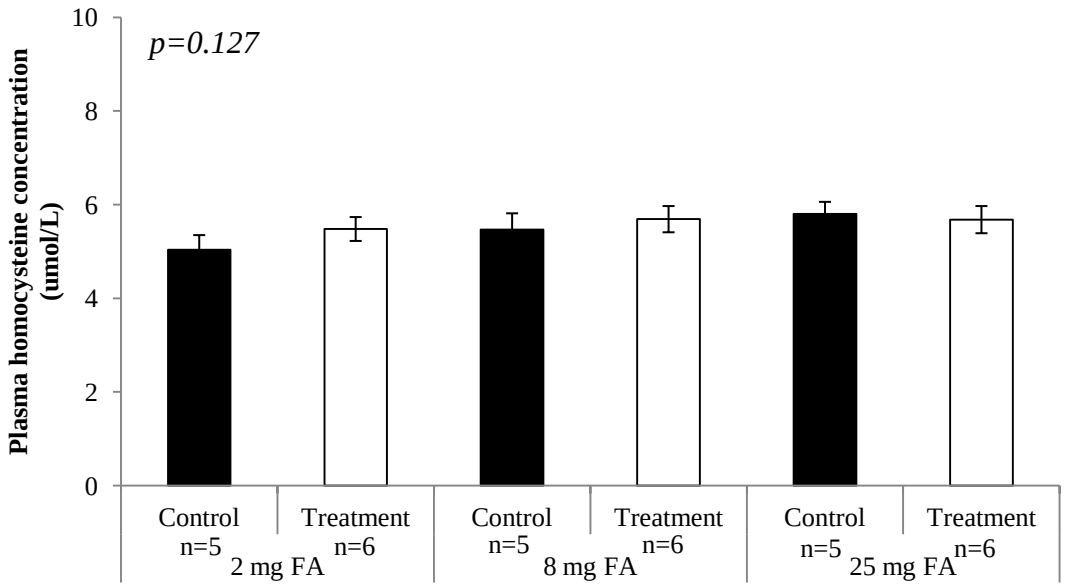

Figure 4.5. The effect of folic acid supplementation and 5-fluorouracil treatment on plasma homocysteine concentrations. Results are expressed as mean ± SEM. P-value denotes a significant main effect of diet at $p \leq 0.05$.

4.4.4. Xenograft growth

There was no independent effect of diet or treatment on xenograft growth (p=0.9 and p=0.2, respectively). There was however a significant interaction effect between diet and treatment (p=0.007). This means that there was an effect of diet which was dependent on the treatment, and vice versa. As expected, the animals on the 2 mg FA diet benefited from treatment, where the relative xenograft growth of the animals receiving treatment were about half that of the animals in the control group (p<0.0001) (**Figure 4.6** and **4.7**). This diet group was the only group to benefit from chemotherapy. In the 8 mg FA group, animals receiving treatment surprisingly fared worse, where treated animals had 1.4 times the tumor size than control animals (p=0.048), suggesting resistance to chemotherapy. There was no effect of treatment in the 25 mg FA group (p=0.3).

Interestingly, there were differences in tumor growth among the untreated animals (**Table 4.7**). The 8 mg FA group had the slowest tumor growth among all untreated animals (p<0.0001).

Without treatment, the 2 mg FA group fared worst compared to the 8 and 25 mg FA groups where tumors were 1.1 and 1.6 times larger, respectively ($p=0.1$ and $p<0.0001$, respectively). Among the treated animals, the 2 mg FA group had the slowest tumor growth ($p<0.0002$), and there was no significant difference between the 8 and 25 mg FA group ($p=0.3$).

Table 4.7. Xenograft growth comparisons between dietary and treatment groups.*

	Ratio of RTV (95% CI)	P-value
Untreated (saline)		
- 2 vs 8	1.14 (0.98-1.33)	0.1
- 2 vs 25	1.64 (1.42-1.88)	<0.0001
- 8 vs 25	0.70 (0.59-0.82)	<0.0001
Treated (5FU)		
- 2 vs 8	0.82 (0.53-1.26)	0.4
- 2 vs 25	0.64 (0.50-0.81)	0.0002
- 8 vs 25	1.29 (0.83-2.01)	0.3

* P-value denotes a significant main effect due to diet at $p \leq 0.05$.

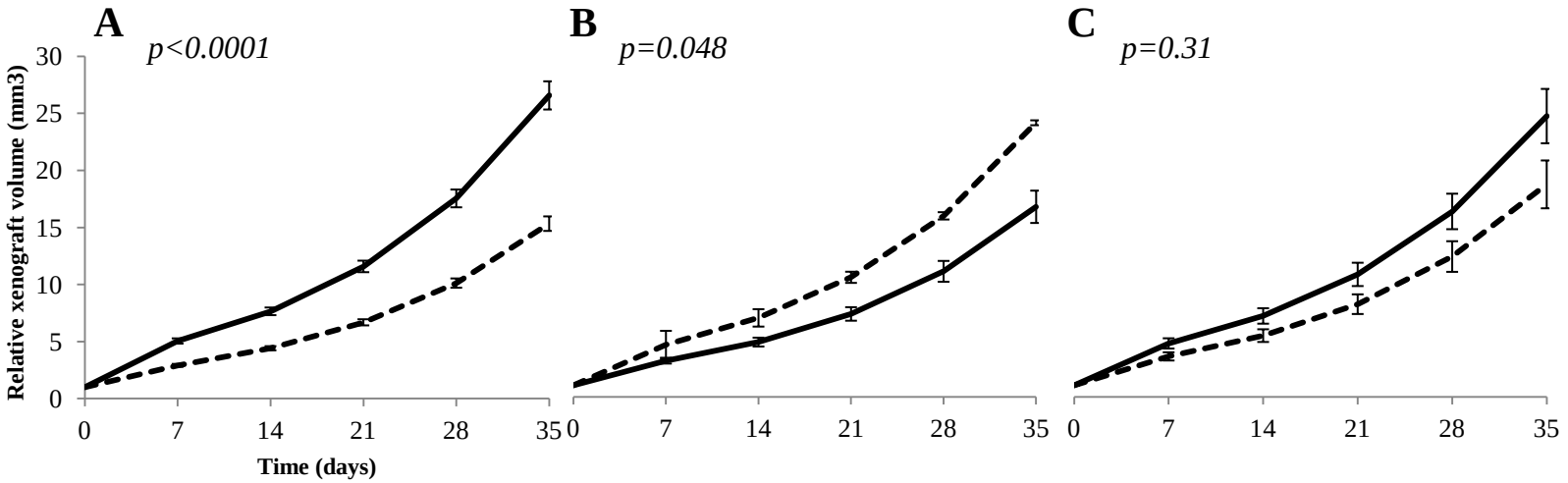

Figure 4.6. The effect of folic acid supplementation and 5-fluorouracil treatment on relative xenograft growth. Animals received one of three diets, 2 (A), 8 (B) or 25 mg FA/kg diet (C) and were administered IP injections of 5FU (dotted), or saline (solid); 2/control: n=8, 2/treated: n=6, 8/control: n=6, 8/treated: n=7, 25/control: n=5, 25/treated: n=6.

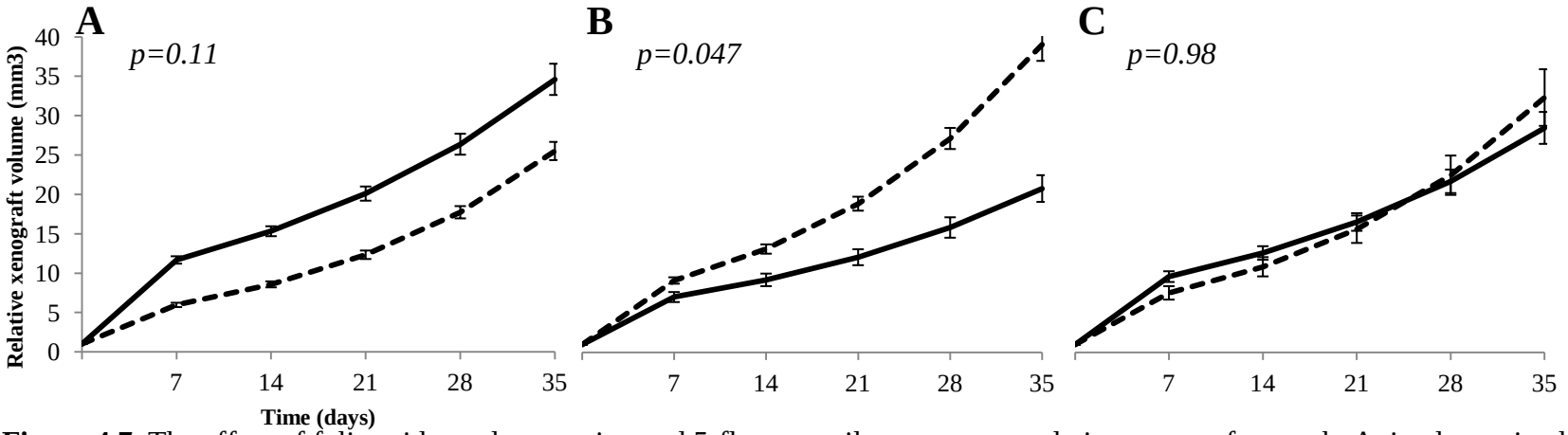

Figure 4.7. The effect of folic acid supplementation and 5-fluorouracil treatment on relative xenograft growth. Animals received one of three diets, 2 (A), 8 (B) or 25 mg FA/kg diet (C) and were administered IP injections of 5FU (dotted), or saline (solid). Relative body weight at time of randomization was added to this model as a covariate; 2/control: n=8, 2/treated: n=6, 8/control: n=6, 8/treated: n=7, 25/control: n=5, 25/treated: n=6.

4.4.5. Gene expression of genes involved in folate metabolism and multidrug resistance

First, gene expressions of TS and DHFR were measured to determine whether FA had modulated intracellular folate metabolism (**Table 4.8**). Fold change in gene expression was measured relative to expression levels found in HCT116 cell culture, for the reason that it serves as a control for all six groups of this study. Fold change in expression of TS was not statistically different among the groups ($p=0.6$). Similarly, there was no significant difference in fold change in gene expression for DHFR ($p=0.8$). Finally, there was no difference in fold change in gene expression in MRP5 or MRP 8 between the groups ($p=0.2$ and $p=0.8$, respectively). Interestingly, among treated animals, regardless of diet group, showed trends of decreased MRP1 expression levels, which did not reach statistical significance ($p=0.08$).

4.4.6. Measures of tumor burden

The mean sum of tumors per tumor-bearing animal was not significantly different among the groups ($p=0.2$) (**Table 4.9**). Similarly, the largest xenograft diameter and mean xenograft volume were not significantly different among the groups ($p=0.7$ and $p=0.7$, respectively). One hundred percent of animals had macroscopically coloured and swollen inguinal lymph nodes indicative of immunological response to the cancer.

There was no significant effect of body weight over the duration of the study ($p>0.05$), but was subsequently added to the regression model as a covariate to determine whether any latency in time to randomization had any significant effect on chemosensitivity (**Figure 4.7**). There was no longer a significant effect of treatment for the 2 mg FA group ($p=0.11$). However, the effect of treatment remained among the 8 mg FA group ($p=0.047$) and there was no effect among the 25 mg FA group ($p=0.98$).

Table 4.8. The effect of folic acid supplementation and 5-fluorouracil treatment on relative fold change in gene expression of enzymes involved in folate metabolism and multidrug resistance, relative to expression levels in HCT116 cell culture.*

Gene	2 mg FA		8 mg FA		25 mg FA		P-value
	Control	Treatment	Control	Treatment	Control	Treatment	
TS							
Mean ± SD (n)	0.24 ± 0.13 (5)	0.28 ± 0.17 (5)	0.22 ± 0.10 (4)	0.21 ± 0.08 (4)	0.27 ± 0.04 (5)	0.18 ± 0.02 (4)	0.66
CV	48%	61%	45%	38%	15%	11%	
DHFR							
Mean ± SD (n)	0.14 ± 0.08 (4)	0.11 ± 0.06 (5)	0.12 ± 0.09 (4)	0.14 ± 0.05 (4)	0.17 ± 0.07 (5)	0.12 ± 0.01 (3)	0.80
CV	57%	55%	75%	36%	41%	8%	
MRP1							
Mean ± SD (n)	3.38 ± 2.83 (3)	0.77 ± 0.44 (4)	2.58 ± 3.71 (4)	1.49 ± 1.07 (4)	2.67 ± 1.02 (5)	0.80 ± 0.26 (5)	0.08
CV	84%	57%	144%	72%	38%	33%	
MRP5							
Mean ± SD (n)	8.05 ± 7.84 (4)	1.21 ± 1.52 (4)	4.49 ± 6.08 (4)	2.41 ± 1.48 (4)	4.96 ± 2.67 (5)	1.60 ± 1.12 (5)	0.16
CV	97%	125%	135%	61%	54%	70%	
MRP8							
Mean ± SD (n)	3.18 ± 4.68 (3)	0.68 ± 1.10 (4)	1.11 ± 1.56 (4)	0.65 ± 0.59 (4)	0.72 ± 0.35 (4)	0.55 ± 0.13 (5)	0.83
CV	147%	162%	141%	91%	49%	24%	

*Results are expressed as relative mean fold change in gene expression ± SD. P-value denotes a main effect of diet ($p<0.05$)

Table 4.9. The effect of folic acid supplementation and 5-fluorouracil treatment on measures of tumor burden.*

Diet	2 mg FA		8 mg FA		25 mg FA		*P* value
Intervention	Control	Treatment	Control	Treatment	Control	Treatment	
Mean sum of tumors per animal	1.3 ± 0.2	1.2 ± 0.12	1.2 ± 0.2	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	0.23
Mean tumor volume (mm^3)	3015.8 ± 0.6	3015.8 ± 0.7	3479.4 ± 0.7	4614.2 ± 0.7	2098.9 ± 0.6	2984.0 ± 1.0	0.72
Swollen lymph nodes (%)	100	100	100	100	100	100	N.S.

*Results are expressed as mean ± SEM. P-value denotes a main effect of diet ($p<0.05$)

4.4.7. Histological evaluation of tumors

All tumors exhibited high-grade, poorly differentiated adenocarcinoma, identified by its loss of tissue architecture, cellular hyperplasia and nuclear hyperchromasia. All viable tumor had mitotic counts greater than 100 mitoses/10 hpf, an indication of actively proliferating cells. Positivity of Ki-67 staining (**Figure 4.8**) was not significantly different among the six groups (**Figure 4.9**). Similarly the degree of necrosis of the tumors was not significantly different among the groups, however animals of the 8 mg FA group have the largest proportion of tumors exhibiting the highest degree of necrosis (**Figure 4.10**).

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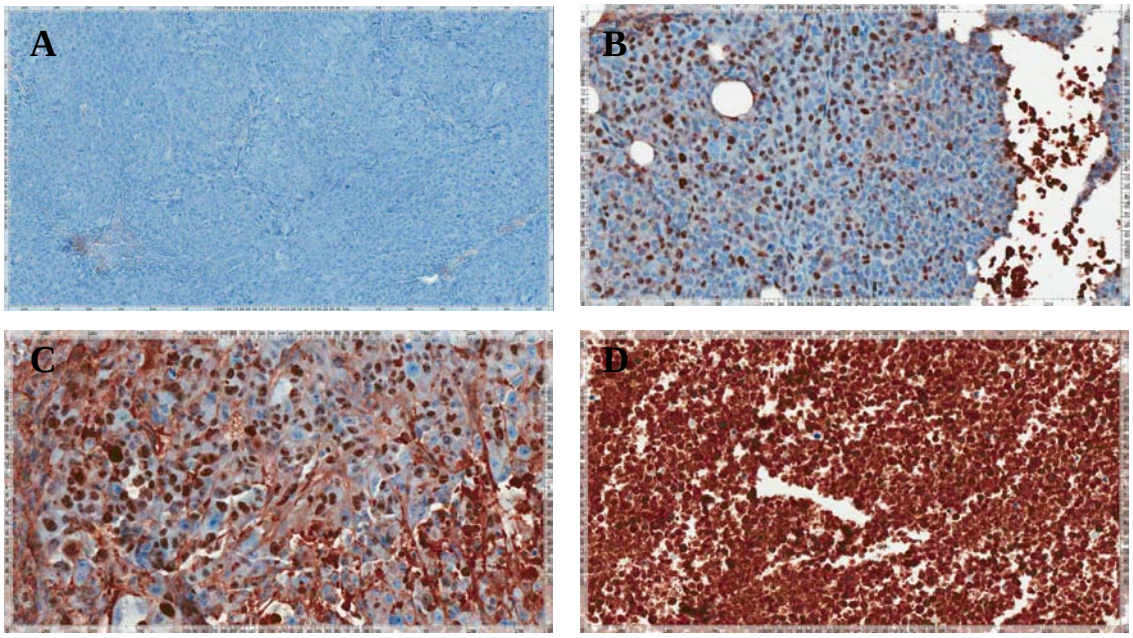

Figure 4.8. Three-stage scoring index of Ki-67 staining. Panels represent 0%(A), 30%(B), 65%(C) and >99%(D) of positive staining.

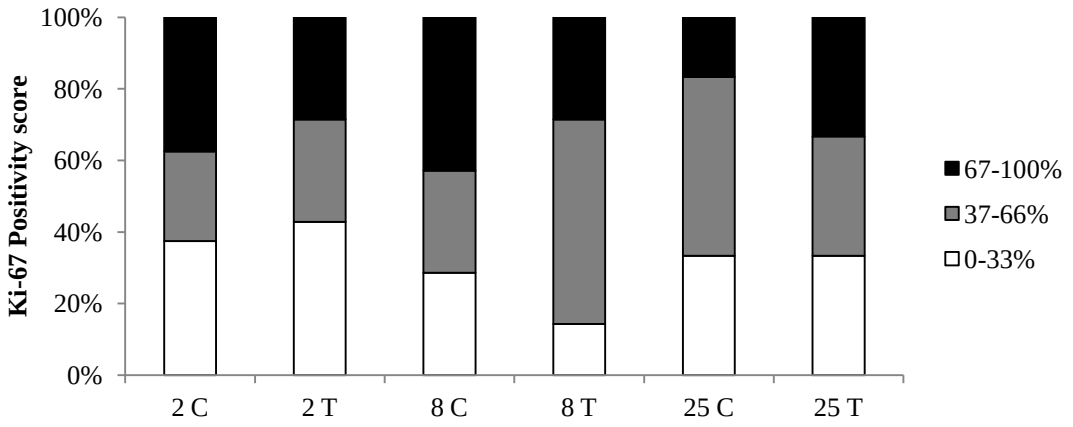

Figure 4.9. The effect of folic acid supplementation and 5-fluorouracil treatment on proliferative marker, Ki-67 in xenograft. Groups are indicated on the x-axis as 2C (2 mg FA untreated; n=9), 2T (2 mg FA treated; n=7), 8C (8 mg FA untreated; n=6), 8T (8 mg FA treated; n=7), 25C (25 mg FA untreated; n=5), and 25T (25 mg FA treated; n=6) (p=0.9).

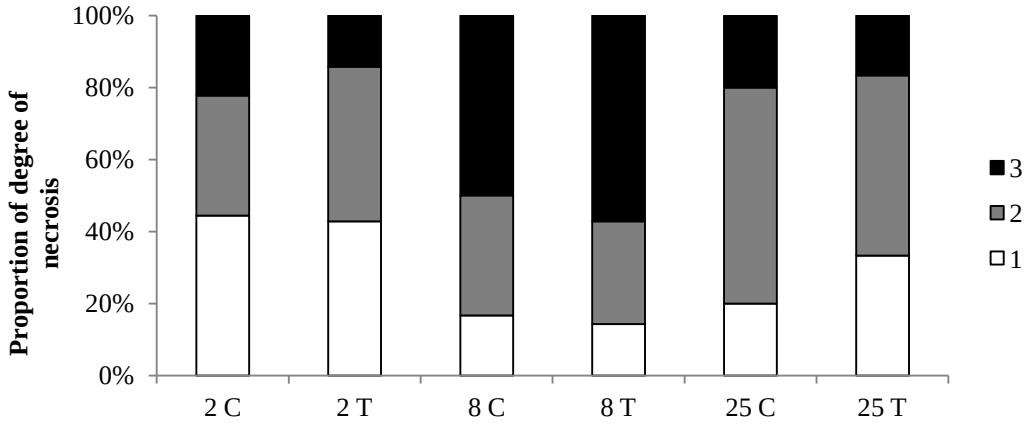

Figure 4.10. The effect of folic acid supplementation and 5-fluorouracil treatment on necrosis in xenograft. Degrees of necrosis are classified as 1 (<33%), 2 (34-66%) and 3 (>67%). Groups are indicated on the x-axis as 2C (2 mg FA untreated; n=9), 2T (2 mg FA treated; n=7), 8C (8 mg FA untreated; n=6), 8T (8 mg FA treated; n=7), 25C (25 mg FA untreated; n=5), and 25T (25 mg FA treated; n=6) (p=0.4).

4.5. Discussion

In the present study, we investigated whether FA supplementation could affect chemosensitivity to 5FU in a xenograft model of human colon carcinoma. Animals in this study began treatment once xenografts were 60-100 mm^3, a range chosen to characterize an established tumor, but prior to its phase of exponential growth [301, 349]. Due to the expectation of a bilateral xenograft development, there was latency in randomization and resulted in animals being randomized based on their unilateral xenograft. Though initial xenograft volume was not statistically different among the groups, the 8 mg FA group had a wider range in size. Diet was initiated in tandem with treatment for the reason that it is unknown whether FA could enhance or hinder xenograft establishment.

The 2 mg FA/kg diet served as the control diet, as this level is considered the rodent BDR for folate [339, 340] and is representative of the RDA of 400 µg for humans. This level was chosen based on the amount of FA expressed relative to caloric content (0.5-1.0 mg per 2000 kcal/day), and its similarity to human intake, and has been used successfully as the control diet in many studies [263-265, 322]. For the purposes of this study, the supplemental levels of FA were chosen based on the daily FA intake seen in humans. The 8 mg FA/kg diet is four times the BDR, intended to reflect four times the RDA. This level is readily achieved by a diet rich in fortified foods in addition to a dietary supplement containing 1 mg FA, totaling 1.6 mg FA/day. The highest level of supplemented diet, 25 mg FA/kg diet was chosen because the extrapolated equivalent to humans (5 mg FA/day) is prescribed to cancer patients in some cases.

In this study, the plasma folate status of the animals is within the range of previously reported rodent studies which had provided comparable levels of 2 and 8 mg FA supplementation [277, 278]. Levels achieved by the 25 mg FA are slightly higher in this study

than that of other studies from our lab [277], as well as unpublished data. Hepatic liver concentrations for animals on the 2 and 8 mg FA diet are similar, to slightly higher, than previous studies [263, 350], however the 25 mg FA is significantly higher than previously observed in our laboratory. The different lobes of mouse liver have varying affinities for metabolites [343], and this was controlled for by using only the left lateral lobe to determine hepatic folate concentrations. Both plasma and hepatic folate did not show a clear dose-dependent relationship with increasing folate suggesting a threshold effect of DHFR at concentrations achieved by the 8 mg FA diet. In addition, treatment had no significant effect on folate concentrations, nor was there an interaction effect between diet and treatment.

There are critical differences in folate metabolism between humans and rodents which must be thoroughly considered. Up to a 35-fold difference in DHFR activity is observed where humans metabolize FA at an extremely slow rate compared to rodents [323]. Therefore, there is no evidence of circulating UMFA in rodents because of the large enzymatic capacity to metabolize FA. Bailey *et al* suggests that clinical trials using high levels of FA are limited by the saturation of DHFR, as supported by UMFA observed in plasma and urine [176, 323]. Moreover, the levels of FA supplementation used in this and other studies may not be sufficient to elicit the same effect in humans, such that rodents may have to be given oral doses with a much greater amount of FA. Therefore it is unlikely that a simple multiplication of human RDA for folate can be linearly adapted to rodent BDR for folate. Evidence suggest that a 10-20 times exposure in rodents may equate to a mere 1-2 times RDA in humans [106].

Dietary FA supplementation was effective in modulating xenograft folate, as concentrations increased in a clear dose-dependent manner, characteristic of the ability of cancer cells to aggressively obtain folate. An interesting observation is that treatment had no effect on

folate concentrations, suggesting that the tumor inhibition seen in the treated 2 mg FA animals cannot be attributed to differences in folate uptake. This is consistent with clinical observations suggesting that a patient's folate status remains stable throughout the course of chemotherapy [294]. In fact, FR is overexpressed on many cancer cells, the leading concept supporting the development of FR-drug conjugates for more targeted therapies [351, 352].

5FU is the cornerstone of CRC chemotherapy, and its primary mechanism of action is the formation of an inhibitory ternary complex with TS [17]. In a rodent study by Branda *et al*, there were dramatic differences in survival among animals receiving high, replete or low folate diets, where the high folate animals demonstrated better survival compared to the latter two groups [292]. However to achieve high folate levels, animals were administered IP injection of a solution containing 50 mg FA/kg in addition to a 2mg FA/kg diet pellet diet [292]. This direct administration of FA likely mimicked the common administration of LV with 5FU in humans, since LV and FA have shown comparable metabolism when administered in this manner [353]. Thus, the present study used three levels of FA supplementation in addition to the doses of LV and 5FU commonly used for non-metastatic CRC in humans. The Mayo Clinic regimen administers an IV infusion of 20 mg/m^2 LV over one hour, followed by an IV bolus of 425 mg/m^2 5FU. There is evidence that dose translation from humans to rodents produces more relevant data if body surface area is used for normalization, instead of body weight [354]. This is for the reason that surface area has shown to better correlate with oxygen utilization, caloric expenditure, basal metabolism, blood volume and renal function [354]. However for the purposes of the present study, we chose to use a dosage that was previously used [unpublished data] to be able to draw comparisons.

This study was the first to demonstrate that there is a potential harmful effect of FA supplementation at levels equivalent to postfortification dietary intake with a daily FA-containing supplement, when combined with 5FU chemotherapy *in vivo*. As expected, the animals of the 2 mg FA responded to 5FU chemotherapy, where treated xenografts were effectively half the size of the controls. However, at higher levels of FA supplementation, sensitivity to 5FU decreased in a non-dose-dependent manner. There was not an independent effect of either diet or treatment. The intermediate range of 8 mg FA/kg diet fared worst in combination with treatment compared to 2 and 25 mg FA groups, where the rate of tumor growth and proportion of necrotic tumor were highest. It is interesting to note however, that the tumor growth of the 8 mg FA untreated group was comparable to that of the 2 mg FA treated group. Though it is counterintuitive and conflicting with current evidence that an expanded intracellular folate pool suppressed tumor growth in actively proliferating CRC cells, it is interesting that in fact the 8 mg FA untreated group had half the size of tumors in the 2 mg FA untreated group.

Xenograft folate concentrations of the 25 mg FA mice were highest compared to the other diet groups. Cancer cells have shown to exhibit a high rate of uptake, high FPGS activity to sequester folate metabolites, low GGH activity, and a low rate of folate efflux, compared to normal cells [355]. High levels of intracellular folate have shown to inhibit DHFR, TS and MTHFR [323, 356, 357]. We speculate that although folate metabolites were taken up by cells, the activities of key enzymes were suppressed and responsible for the null effect of treatment in the 25mg FA xenograft samples, compared to the 2 and 8 mg FA mice.

Evidence suggest that intracellular folate can in fact modulate chemosensitivity by inducing TS expression [79, 308], competitively inhibit antifolates from polyglutamylation [314,

315], and recently, upregulate efflux activity of membrane transporters capable of binding drug metabolites [19, 96].

To elucidate whether there were changes in folate metabolism between groups, gene expression analyses of TS and DHFR were carried out in this study. FA must be reduced by DHFR before it can act as a cofactor [151]. In humans, high levels of FA supplementation can saturate DHFR enzyme activity, increasing intracellular DHF levels and thereby suppressing MTHFR and TS activity [151, 179]. In addition, DHFR is downregulated at high folate levels as a method of maintaining intracellular homeostasis [358], but our analyses did not reflect this regulation. This difference in response may be due to the large DHFR capacity in rodents, unable to reach a point of enzyme saturation. In this study, there was no apparent change in TS or DHFR activity among any diet or treatment group, compared to what is expressed in HCT116 cell culture. There is evidence that TS activity is better detected at the post-transcriptional level [359], which may not be captured by mRNA expression analysis. Furthermore, high intracellular folate concentrations have been shown to increase the concentration of the TS ternary complex, and induce loss of transcriptional control, thereby increasing the number of binding sites for free 5,10-methyleneTHF [305, 306, 308]. In addition, repeated cycles of 5FU also increase TS activity [79]. However, the evidence supporting these ideas stem primarily from *in vitro* studies, which expose cells to levels well beyond human relevance. Therefore, we found that DHFR and TS are not sensitive to changes in intracellular folate concentrations achieved by FA supplementation at 2, 8 and 25 mg FA/kg diets, and are similar to that of cells growing in unsupplemented media. Moreover, there is no evidence that there were changes in folate metabolism as regulated by TS or DHFR. Therefore, we were unable to confirm whether the increasing concentration of xenograft folate were due to changes in enzymatic activity and could

explain the non-dose dependent response to 5FU, particularly the null effect in the 25 mg FA mice.

Our immunohistochemical analyses were unable to elucidate relationship between cellular proliferation, programmed cell death and necrosis. Cancer cells with highly actively proliferating cells have lower levels of pro-apoptotic activity, compared to normal cells. The relationship between Ki-67staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is an effective method of determining the rate of proliferative activity in cancer cells. In the present study, we were unsuccessful in TUNEL and could not include apoptosis data in our xenograft samples. Necrosis is a passive cellular process that fails to capture the rate of cancer cell proliferation. Therefore, our immunohistochemical data on cell proliferation and apoptosis and histological data on necrosis are difficult to interpret. The 8 mg FA mice showed the largest proportion of Ki-67 positive cells, however the same samples also showed the highest proportion of necrosis. The combination of these observations fails to indicate the balance between pro- and anti-apoptotic processes, which are key factors in response to chemotherapy.

MRPs responsible for antifolate and antimetabolite drug efflux have been implicated in folate homeostasis based on three lines of evidence. Firstly, there were significantly diminished pools of intracellular folate in MRP-overexpressing cells [96]. Second, MRP overexpression increased folate-dependent cellular growth after a 4-hour exposure to folate [360]. And lastly, MRP expression is downregulated at the protein level in folate-restricted conditions [360]. Hooijberg *et al* [360] demonstrated in an ovarian carcinoma cell line that both folate and antifolate efflux increases in higher folate environments in an MRP1-upregulated system. However this finding was not confirmed in our xenograft samples. MRP1 expression varied

greatly, and we were unable to draw any firm conclusions. Next MRP5 was interrogated since it has been shown to export 5FU metabolites [78] and folates [91]. Weilinga *et al* showed in an human embryonic kidney cell line that MRP5 mediate transport antifolates and folates which may confer resistance to drugs [91]. Again, this finding was not confirmed among our samples, and there was too much variation among the groups. Finally, MRP8 was interrogated, for the reason that it has shown to strongly correlate with 5FU efficacy [92]. In an *in vitro* study of small-cell lung cancer line, Oguri *et al* [92] showed that MRP8 actively pumps out FdUMP, the main active metabolite of 5FU, and increases in transcription could likely confer resistance. Thus, MRP8 was interrogated in this study to elucidate whether it played an important role in chemosensitivity, particularly among the 8 and 25 mg FA animals, however our data did not reflect this. Furthermore, MRP8 was not selectively upregulated in treatment animals. To our knowledge, the present study is the first to investigate MDR in an *in vivo* system. A recent *in situ* study of removed human colon carcinoma shows up-regulated, down-regulated and no change in MRP1, MRP5, and MRP8 expressions, respectively, compared to control tissues [105]. In the present study, there was too great of variability among the samples, particularly in the 8 mg FA group, to draw hard conclusions. It is unknown whether the variation in initial xenograft volume could be solely responsible for the variation seen in the group, or whether diet had a supporting role. To our knowledge, this is the first study to investigate MRP activity outside of an *in vitro* or *in situ* environment, which could explain the large variability. Further *in vitro* characterization of MRP activity with the HCT116 colon carcinoma cell line will provide a general understanding of the mechanisms of MDR to 5FU in this particular cell line. Specifically, investigating the effects of MDR activity in folate-depleted, -repleted or –supplemented media would formulate the necessary hypotheses for a subsequent *in vivo* study. This is the first study which sought to

investigate the relationship between physiologically relevant levels of FA supplementation and chemosensitivity to 5FU *in vivo*, and investigate mechanisms of folate-induced resistance.

There are other mechanisms of resistance that may have contributed to the decrease in chemosensitivity. In addition to the efflux of anticancer agents, the blocked uptake or influx and increased metabolism have been attributed to play a role. In fact, there is evidence that DPD activity can increase following repeated cycles of 5FU, resulting in increasing doses to elicit the same level of cytotoxicity *in vitro* [311, 361, 362]. There is large interindividual variability in hepatic DPD activity in humans, which has a profound effect on response to 5FU chemotherapy[363]. In addition, altered anticancer targets, altered cell-cycle checkpoints and cancer cell proliferation are valuable manipulations induced by oncogenic processes [78]. However, in this study, only 5FU efflux was explored since MDR proteins have shown sensitivity to intracellular folate concentrations.

Considering the high degree of necrosis we observed among all six groups, it is possible that any effect of diet or treatment was overridden by the aggressiveness of the cancer. This particular cell line is highly proliferative due to its Ras+ mutation and *in vitro*, requires medium renewal every two to three days [328]. HCT116 human colon carcinoma exhibits MSI phenotype with wildtype p53 function. Therefore, its neoplastic transformation is triggered by a defective MMR system, which has been shown to exhibit some resistance to 5FU alone, relative to other CRC phenotypes. It would be interesting to investigate the relationship of the effect of FA supplementation on chemosensitivity to 5FU in a cell line that exhibits CIN, a phenotype that has shown greater response to 5FU treatment, such as HT29. HCT116 was chosen over HT29 for its rapid cell proliferation, aggressiveness and has been successfully propagated in a FA-containing medium in our laboratory. Given the high proliferative rate and cell turnover of this cell line, it

was surprising that xenograft establishment was ineffective, resulting in a much smaller sample size.

Due to inefficient xenograft establishment, only 41 xenograft samples were obtained and anticipation of bilateral xenograft growth resulted in larger tumors at the time of randomization. Although this athymic model is immunocompromised, the genetic heterogeneity as a result of its outbred background may be responsible for the failure of tumor take. Previous studies in our laboratory used an inbred *nu/nu* mouse model, which exhibited 100% tumor take; however was not able to withstand repeated doses of 5FU without succumbing to toxicity-related side effects [301]. In the present study, animals were mostly randomized based on their unilateral xenograft once it was evident the second would not develop. The latency may have affected chemosensitivity to 5FU. Since xenograft growth plateaus following an exponentially proliferative stage, it is presumed ideal to begin intervention prior to this phase for maximal incorporation of 5FU metabolites into cancer cells. As such, it is possible that this mark was missed and in some cases the larger xenografts did not respond to treatment. The 8 mg FA group had consistently worse outcomes compared to the other diet groups, in xenograft growth and accordingly, proliferation and necrosis. This group had the smallest proportion of Ki-67 positive staining less than 33% and had the greatest proportion of necrotic tissue. In both measures, the effect of treatment was not evident. Future studies to confirm these findings should be cautious of the xenograft size at randomization, such that each animal bears the xenografts less than 100 mm^3.

A sample size of 150 (25 mice per group) was determined to be sufficient to detect a difference of 0.65 standard deviations in xenograft growth between any level of diet, with 80% power. Due to ineffective xenograft growth, only 34 animals were randomized into diet and

treatment groups, resulting in a underpowered sample size. The effect size of diet and treatment in the 2 and 8 mg FA group was large enough that we were still able to detect a significant effect at alpha levels $p \leq 0.05$. However, there was no effect in the 25 mg FA group. Thus, findings of this study should be carefully interpreted, given that the study was underpowered.

The intermediate diet of 8 mg/kg diet is physiologically relevant to humans, intended to represent a level achieved by a diet rich in fortified foods with a daily 1 mg FA supplement. A large proportion of cancer patients in fortified countries take dietary supplements which contain a minimum of 400 μg FA [173]. Although there is no firm evidence supporting the benefit of dietary supplements, individuals continue to take supraphysiological levels of FA for their potential, yet unfounded, health benefits. Furthermore, 5FU is an antimetabolite which inhibits an important enzyme involved in folate metabolism, is the core anticancer agent for all CRC chemotherapy. Given the growing body of *in vitro* evidence that high levels of FA may potentially interfere with 5FU efficacy, this study explored the adverse effect of FA supplementation on chemosensitivity. Thus the findings of this study demonstrated that the large population of cancer patients may be interfering with their own treatment, by supplementing their FA-fortified diet with dietary supplements which disrupt chemotherapeutic efficacy. Furthermore, antifolate and antimetabolite drugs are not only reserved for the treatment of cancers, but are used in the management of other health conditions as well, such as rheumatoid arthritis (RA).

MTX, an inhibitor of DHFR, is a classic antifolate used in the treatment of cancers, autoimmune diseases and termination of pregnancies. Commonly used for RA, the primary mechanism is not the inhibition of DHFR, but rather the inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICART), which inhibits purine synthesis, resulting

in an accumulation of adenosine which exhibits an anti-inflammatory effect via interaction with receptors on neutrophils and mononuclear cells [364, 365]. FA supplementation of up to 5 mg FA/week is prescribed to suppress common side effects which often lead to premature termination of treatment, since folate deficiency can increase the toxicity of MTX [366-368], and FA supplementation allows the use of higher MTX dosing [369]. Given its mechanism of action there is a theoretical risk that high FA supplementation may reduce the efficacy of MTX, although there is little supporting evidence [367, 370, 371]. On the other hand, a recent study of MTX dosage suggests that higher MTX dosing is required to elicit the same pharmacological response in the post FA fortification era, compared to dosing prior to fortification, suggesting a decrease in MTX efficacy for RA treatment [372]. Mean annual MTX dose was stable between 1988 and 1996, however a linear increase was observed from 1997 to 1999, a 34% increase ($p<0.001$) [372]. Considering that the general population commonly use dietary supplements, it is necessary to determine whether FA supplementation can suppress antifolate efficacy.

Though, FA fortification is considered a public health success in the prevalence of NTDs, the resultant folate levels achieved through intake of fortified foods and dietary supplement use is unprecedented. Folate deficiency is nonexistent in Canada, and over half of the population regularly uses a dietary supplement containing a minimum of 400 μg FA, the current RDA. Over 50% of cancer patients use dietary supplements through their chemotherapy, of which its mechanisms are based on the interruption of folate metabolism. Furthermore, FA supplementation has been associated with other adverse health outcomes such as masking of vitamin B12 deficiency, accelerated cognitive decline in individuals with low B12 status, decreased natural killer cell cytotoxicity and cancer [151, 176]. In addition, FA supplementation may decrease efficacy of anti-inflammatory drugs, anti-seizure medications and antifolate

chemotherapy [151, 176]. Given the widespread systemic exposure to high FA, this poses an unexpected and immediate need to understand the effects of high FA supplementation for the maintenance of overall health, treatment of diseases using antifolates and antimetabolites, and future public health initiatives. This study was the first of its kind to demonstrate that FA supplementation can decrease the efficacy of 5FU chemotherapy and warrants further investigations to elucidate possible mechanisms.

CHAPTER 5: THE EFFECT OF FOLIC ACID SUPPLEMENTATION ON CHEMOSENSITIVITY TO 5-FLUOROURACIL IN A XENOGRAFT MODEL OF HUMAN COLON CARCINOMA – CONFIRMATION STUDY

5.1. Introduction

In the previous study, we were able to demonstrate a potential harmful effect of FA supplementation on chemosensitivity to 5FU, at a dose physiologically relevant to humans. Given the mechanisms of 5FU, the dramatic increase in erythrocyte folate concentrations may interfere with the efficacy of treatment, particularly among cancer patients who use dietary supplements and consume foods rich in FA. Despite the little to zero evidence supporting any benefit to taking supplements, over 50% of cancer patients adopt or continue dietary supplement use without physician consults [9, 10, 170, 173]. Since FA fortification, folate status has improved dramatically and the surpassed the anticipated increases of 100-200 μg/day [162, 163]. There is a growing body of evidence that FA supplementation may cause adverse health outcomes, and may affect efficacy of antifolate and antimetabolite chemotherapy. Thus, this study investigated whether expanded intracellular folate pools as a result of FA supplementation could affect chemosensitivity of colon cancer cells to 5FU chemotherapy and confirm the results of the previous study with a larger sample size.

5.2. Specific objectives

To confirm the findings of the previous study demonstrating a non-dose dependent decrease in chemosensitivity to 5FU in mice ingesting the 8 and 25 mg FA diets compared to mice ingesting 2 mg FA (control).

5.3. Materials and methods

5.3.1. Animal model

Athymic nude mice from (Harlan Laboratories, Madison, WI, USA) were used, as previously described in **Chapter 4.3.1.**

5.3.2. Experimental design

Male athymic nude mice (Hsd: Athymic nude-Foxn1^{nu}) at four weeks of age, were acclimated for one week during which they received free access to water and an amino acid-defined diet (Dyets, Bethlehem, PA, USA) containing 2 mg FA/kg [336] (**Figure 5.1**). After one week, they were injected subcutaneously with 1.0x10^6 HCT116 human colon carcinoma cells in each flank. Cell suspensions were combined with an equal volume of Matrigel (BD Biosciences) [373, 374]. Approximately 10 days later, established xenografts were 60-100 mm^3 in size and animals were randomized to one of three diet groups, 2, 8 or 25 mg FA/kg diet. Within these groups, animals were further randomized to receive an IP injection of either saline vehicle (0.9% NaCl, 100 µL) or 5FU (20 mg/kg body weight, 100 µL) + leucovorin (1 mg/kg body weight, 100 µL) for five consecutive days. Xenograft dimensions were measured by a digital micrometer caliper three times a week, and graft volumes were calculated as V= ½ (LW^2), where W is defined as the largest measurement, and L is defined perpendicular to W [337, 338]. All animals were housed in sterile polycarbonate microinsolators (1-3 per cage) at a temperature (24 ± 2^{o}C) and humidity (50%) controlled, negative pressure environment, with a 12-hour light/dark cycle. Cages, bedding and environmental enrichment were changed on a biweekly basis, in which all materials were autoclaved-sterile. The use of wire-bottomed stainless steel cages to prevent coprophagy was not used due to ethical considerations. Animal care and use were in accordance

with the Canadian Council on Animal Care 1984 guidelines. This animal protocol was approved by the Animal Care Committee at the University of Toronto, Toronto, ON (Protocol 20009098).

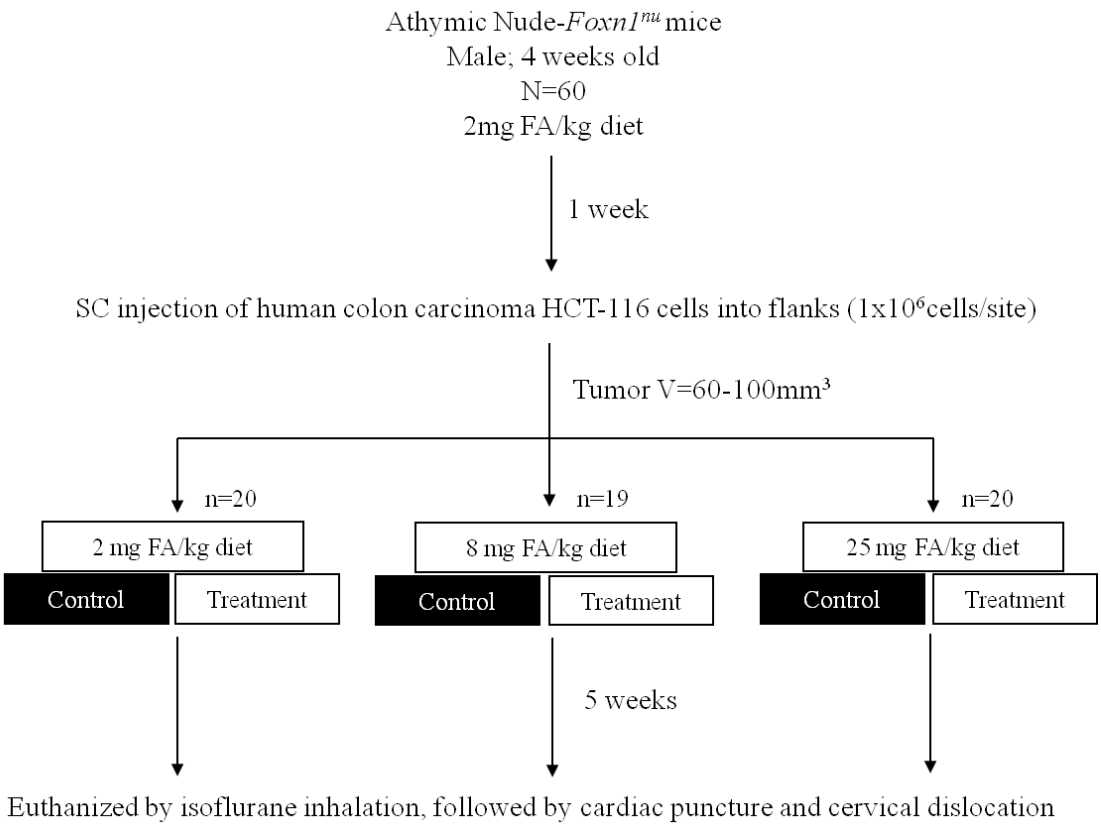

Figure 5.1. Study design. Athymic nude mice were injected with HCT116 human colon carcinoma, subcutaneously in each flank. Once xenografts reached 60-100 mm^3, they were randomized into 2, 8 or 25 mg FA/kg diets. Within each diet group, they were further randomized to saline or treatment (20 mg/kg 5-fluorouracil + 1 mg/kg leucovorin) by intraperitoneal injection for five consecutive days. Xenografts were measured three times a week by digital micrometer caliper, and animals were euthanized 5 weeks following randomization to treatment groups.

5.3.3. Diets

Gamma-irradiated sterile diets in pellet form were used (Dyets, Bethlehem, PA, USA), as previously described in **Chapter 4.3.3**.

5.3.4. Cell culture

Human colon carcinoma HCT116 cells were used (American Type Culture Collection, Manassas, VA, USA), as previously described in **Chapter 4.3.4.**

5.3.5. Xenograft establishment in nude mice

Confluent dishes were washed three times with PBS (Invitrogen, Gaithersburg, MD, USA) and treated with trypsin for 10 minutes in 37^{o}C. Cells were suspended in serum-free media and diluted to 5x10^{6} cells/mL, and combined with an equal volume of Matrigel (BD Biosciences). Four hundred µL of cell suspension solution was immediately administered by subcutaneous injection into each flank of each mouse by a hired animal care technician. The flank was chosen preferentially over the shoulder for ease of handling, as well as a superior take rate and less hindrance of mobility. Xenografts were established within ten days post injection, and randomized to one of six groups (**Figure 5.1**) once xenograft volume was 60-100 mm^{3}.

5.3.6. Drug preparation and administration

LV (Sigma, F7878, powder) and 5FU (Sigma, F6627, powder) was prepared and administered as previously described in **Chapter 4.3.6.**

5.3.7. Sample collection

The sample collection was performed as outlined in **Chapter 4.3.7.**

5.3.8. Determinations of plasma, liver and xenograft folate concentrations

Plasma, liver and xenograft folate concentrations were determined as previously described in **Chapter 4.3.8.**

5.3.9. Statistical analyses

A sample size of 60 animals, 10 per group, was required to be 80% certain of detecting a difference of 1.0 standard deviations, in xenograft growth between any level of diet. This level was chosen based on the spread of data of xenograft growth, of the previous study.

The study design was based on two factors, diet and treatment, and thus a two-way analysis of variance (ANOVA) with the inclusion of an interaction term was used to analyze the following parameters: body weight, initial xenograft volume, folate and homocysteine concentrations, and measures of xenograft burden (mean sum and volume). Prior to testing, dependent variables were tested for normality with a histogram. As well, Levene's test of equality of error variances was not significant, thus meeting the assumptions required to utilize the ANOVA model for statistical testing. If a significant interaction (alpha level $p \leq 0.05$) was observed, Tukey's post-hoc test was performed. Xenograft growth is a continuous variable with increasing scatter in data as the study progressed, violating the assumptions of an ANOVA model. In this case, a generalized estimating equation analysis was used. This statistical testing is appropriate for xenograft growth because it assumes that measurements are correlated with one another. An autoregressive correlation was used because it assumes that measurements taken closer in time from the same animal are more strongly correlated than measures taken further apart in time.

Statistical tests were performed using SPSS 20.0 for PC (IBM, Armonk, New York) and graphs were prepared in Microsoft Excel 2007 and SPSS Output. All statistical tests were two-sided and considered significant at alpha level $p \leq 0.05$.

5.4. Results

5.4.1. Sample size and growth curves

Growth curves were similar among the animals prior to and after randomization. Animals developed bilateral xenografts by day 10 post injection and were randomized into each of the diet groups. This resulted in 19 to 20 animals per diet group. The addition of Matrigel ensured effective xenograft establishment within 14 days. Thus neither body weight nor initial xenograft volume was statistically different among the six groups (**Table 5.1**).

Unlike the previous study, there was a difference in weight gain between the control and treatment groups, where the former were on average 0.89 g heavier, regardless of diet ($p=0.074$). But there was no evidence that this difference changed over time.

5.4.2. Folate concentrations

Plasma folate concentrations exhibited FA dose dependence and was statistically different among the diet groups ($p<0.001$), but treatment had no effect ($p=0.8$) (**Figure 5.2**). The 2 mg FA diet group has significantly lower plasma folate concentrations compared to the 8 and 25 mg FA groups, $p\leq0.05$ and $p<0.0001$, respectively. The latter two diet groups were also statistically different from each other, where the animals of the 25 mg FA diet group had the highest concentrations of plasma folate ($p<0.0001$). Surprisingly, hepatic folate concentrations were not significantly different among the diet groups ($p=0.53$), nor did treatment have an effect ($p=0.18$) (**Figure 5.3**). On the other hand, xenograft folate concentrations increased dose dependently with FA supplementation ($p=0.002$) (**Figure 5.4**). The 2 mg FA group had significantly lower folate concentrations compared to the 8 and 25 mg FA groups, $p=0.007$ and $p=0.001$, respectively. However the latter two were not statistically different from each other ($p=0.54$).

Table 5.1. Baseline characteristics of animals at the time of randomization.*

Diet	2 mg FA		8 mg FA		25 mg FA		*P* value
Intervention	Control	Treatment	Control	Treatment	Control	Treatment	
Initial tumor V (mm^3)	91.9 ± 5.5	73.6 ± 5.7	76.9 ± 5.2	76.7 ± 6.9	75.7 ± 6.2	75.9 ± 5.8	0.43
Initial body weight (g)	23.5 ± 0.5	24.6 ± 0.3	24.1 ± 0.6	23.7 ± 0.6	24.1 ± 0.6	23.5 ± 0.5	0.90

*Results are expressed as mean ± SEM. P value denotes a main effect due to diet.

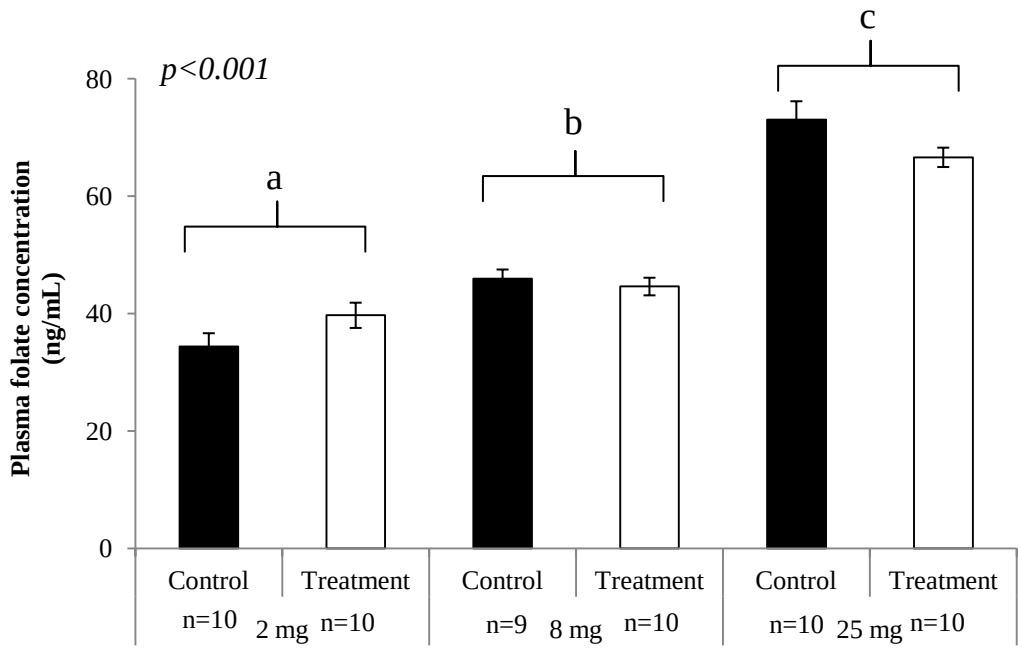

Figure 5.2. The effect of folic acid supplementation and 5-fluorouracil treatment on plasma folate concentrations. Results are expressed as mean ± SEM. P-value denotes a main effect of diet. Means statistically different between diet groups are denoted with different letters ($p \leq 0.05$).

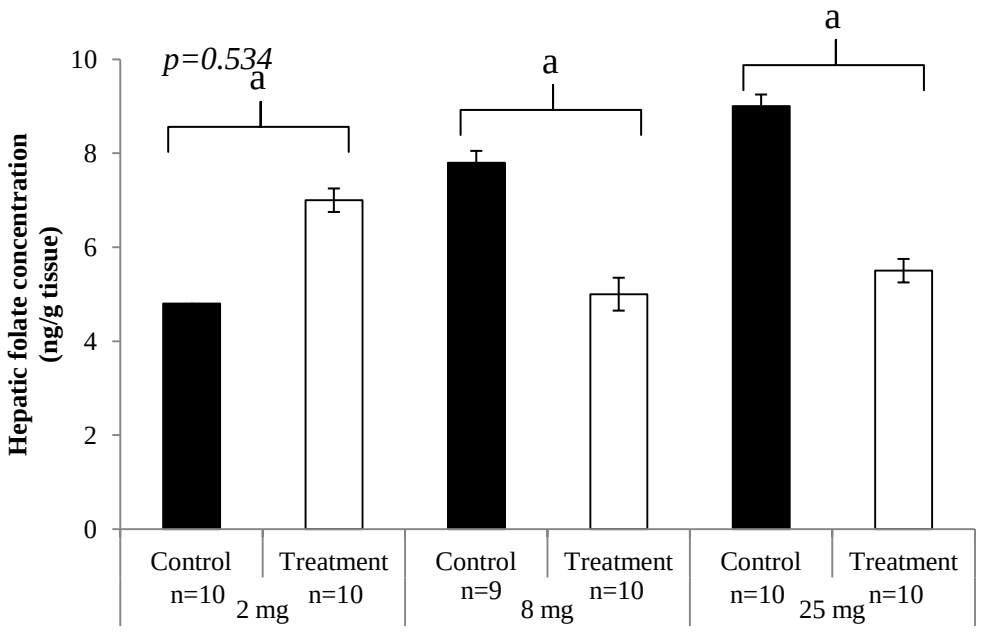

Figure 5.3. The effect of folic acid supplementation and 5-fluorouracil treatment on hepatic folate concentrations. Results are expressed as mean ± SEM. P-value denotes a main effect of diet. Means statistically different between diet groups are denoted with different letters ($p \leq 0.05$).

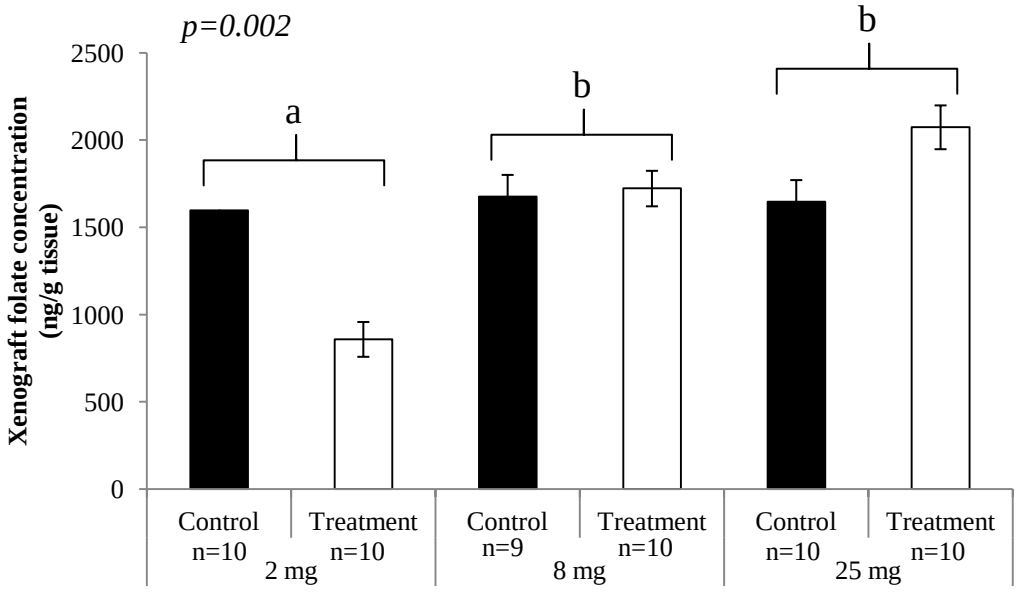

Figure 5.4. The effect of folic acid supplementation and 5-fluorouracil treatment on xenograft folate concentrations. Results are expressed as mean ± SEM. P value denotes a main effect of diet. Means statistically different between diet groups are denoted with different letters ($p \leq 0.05$).

5.4.3. Xenograft growth

In this dataset, there was an independent effect of treatment (p=0.0036), but not diet (p=0.9). Unlike the previous study and contradictory to our hypotheses, the 2 mg FA group did not respond to treatment, in fact, those receiving treatment appeared to have worse outcomes, though it did not reach statistical significance (p=0.076) (**Figure 5.5**). Similar to the previous study, animals of the 8 mg FA diet fared worse on treatment (p=0.043), and there was no effect of treatment for animals receiving the 25 mg FA diet (p=0.9). Since there is evidence that body weight may have had an effect on xenograft growth, we added relative weight as a predictor in the model (**Figure 5.6**). With this, the effect of treatment strengthened (p=0.0005) and all diet groups fared worse on treatment, though this difference was not statistically different.

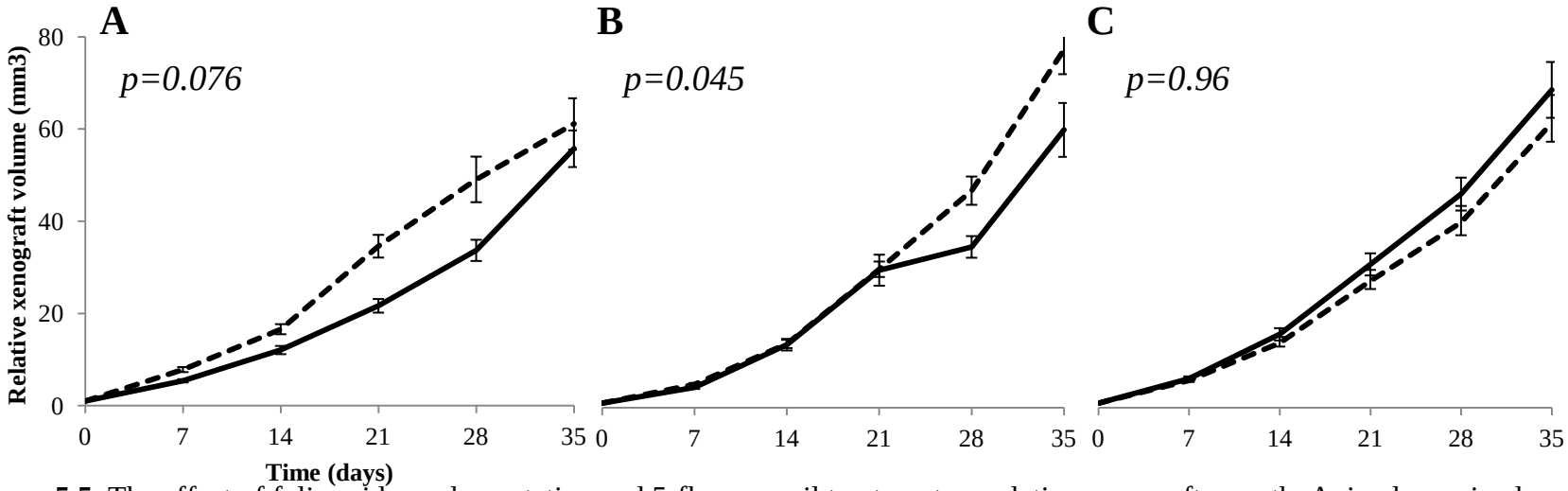

Figure 5.5. The effect of folic acid supplementation and 5-fluorouracil treatment on relative xenograft growth. Animals received one of three diets, 2 (A), 8 (B) or 25 mg FA/kg diet (C) and were administered IP injections of 5FU chemotherapy (dotted), or saline (solid); 2/control: n=20, 2/treated: n=20, 8/control: n=18, 8/treated: n=20, 25/control: n=20, 25/treated: n=20.

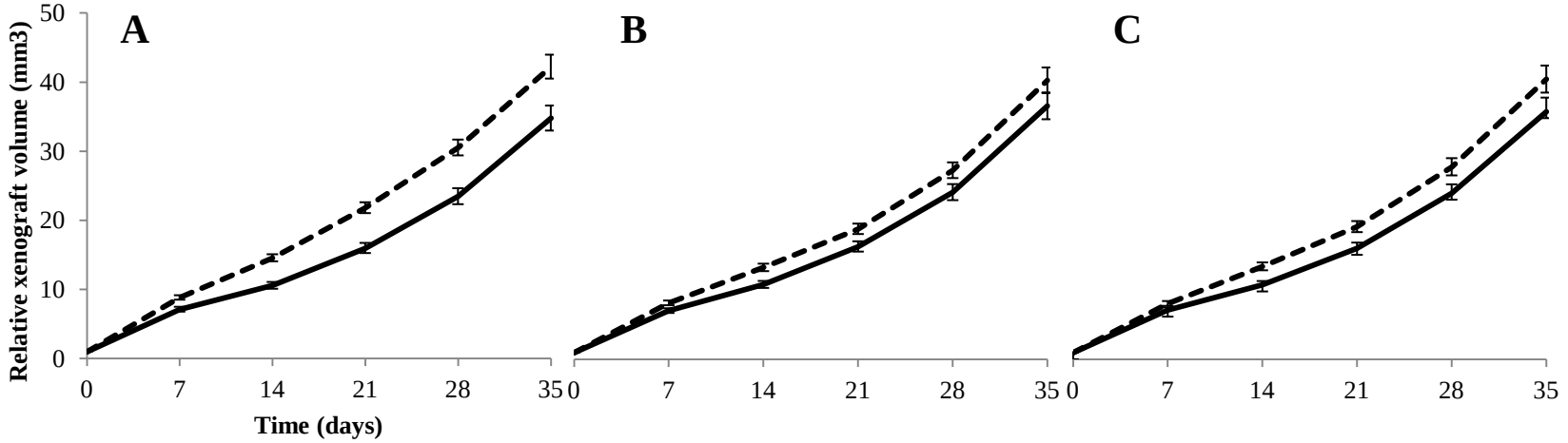

Figure 5.6. The effect of folic acid supplementation and 5-fluorouracil treatment on relative xenograft growth. Animals received one of three diets, 2 (A), 8 (B) or 25 mg FA/kg diet (C) and were administered IP injections of 5FU chemotherapy (dotted), or saline (solid); 2/control: n=20, 2/treated: n=20, 8/control: n=18, 8/treated: n=20, 25/control: n=20, 25/treated: n=20. Relative body weight at the time of randomization was added to this model as a covariate.

Table 5.2. The effect of FA supplementation and treatment on measures of tumor burden.*

Diet	2 mg FA		8 mg FA		25 mg FA		*P* value
Intervention	Control	Treatment	Control	Treatment	Control	Treatment	
Mean sum of tumors per animal	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	N.S.
Mean tumor V (mm^3)	4119.1 ± 0.6	3911.1 ±0.5	3452.2 ± 0.6	5636.0 ± 0.4	3557.1 ± 0.5	3354.3 ± 0.6	0.43

*Results are expressed as mean ± SEM. P-value denotes a significant main effect due to diet at $p<0.05$.

5.4.4. Measures of tumor burden

All animals randomized (n=59) beared bilateral xenografts. Mean tumor volume at the time of necropsy was not significant among the groups (p=0.4), however was significantly larger compared to the previous study (p=0.012) (**Table 5.2)**.

5.5. Discussion

Several *in vitro* studies support the biologically plausible mechanism that FA supplementation may interfere with 5FU metabolites and decrease chemosensitivity [97, 319, 360]. However, few animal studies, to date, have examined whether FA supplementation can affect chemosensitivity to 5FU [292, 293]. Thus, our primary aim was to investigate whether FA supplementation can interfere with response to 5FU chemotherapy *in vivo*. Based on the provocative findings of the previous study where the intermediate level of FA supplementation decreased chemosensitivity, this confirmation study was carried out with a larger sample size to further confirm the results.

Similar to the previous study, dietary FA was effective in modulating plasma folate concentrations, where there was a dose-dependent response with increasing levels of FA supplementation. Hepatic folate concentrations, on the other hand, did not reflect any effect of diet or treatment. Among the control animals, there is a trend of dose dependence to FA, where higher dietary levels are reflected in the liver. However, any differences did not demonstrate statistical significance. Hepatic folate are considered an indication of long term folate status, whereas plasma folate is considered short term because of its daily flux due to dietary intake. It is unknown whether hepatic folate was increasingly shuttled out into the bile and epithelia, resulting in a larger xenograft volume. In the current study, xenografts were significantly larger

and had almost twice the folate concentration, compared to the previous study. Xenograft folate concentration demonstrated trends of dose-dependence, but groups were not statistically different.

The present study was carried out to confirm the suppressive effects of FA supplementation on response to 5FU, evident from the previous study. However, animals receiving chemotherapy demonstrated larger tumor growth compared to animals receiving saline, regardless of FA diet. This difference in response was statistically different in the 8 mg FA group, where treated animals had a significantly higher rate of tumor growth, consistent with previous study findings.

There are possible mechanisms that could explain this unexpected detrimental effect of treatment at all levels of diet. Firstly, to enhance xenograft establishment, Matrigel was added to cell suspension. The addition of this soluble membrane matrix proved to be too robust for this study and xenografts did not respond as predicted to 5FU chemotherapy. Second, 5FU and LV dosage is administered relative to their body weight. Overall, animals had similar body weights at the time of randomization, however in the present study, animals received treatment at a slightly younger age due to the rapid establishment of the xenograft. Therefore, animals bearing bilateral xenografts, essentially bear tumor burden that is twice that of the previous study though drug dosage remained the same. Animals receiving treatment consistently had larger tumors compared to those receiving saline, suggesting the dosage was no longer appropriate given the tumor volume, and the stress associated with drug administration likely played a role in their diminishing health. In fact, even animals receiving the control diet (2mg FA) were unresponsive to treatment, substantiating our contention that the dosage was not appropriate for the increased tumor burden. Indeed, the rate of xenograft growth was significantly higher in the present study than that of the last ($p=0.001$). There was no effect of diet ($p=0.9$), but there was a significant

effect of treatment (p=0.018). This was primarily influenced by the strong negative effect of treatment in the present study.

Treatment of colon carcinoma xenografts with 5FU chemotherapy is a well established model of human CRC. There is a large body of evidence validating the effectiveness of 5FU in nude mouse models in intention-to-treat studies [375-378]. Regression of tumor growth is correlated with elevated intracellular concentrations of FdUMP, FdUTP and FUTP [378-380]. Though there may be differences in 5FU catabolism between rodents and humans, results from rodent studies have consistently demonstrated the validity of xenografts of human tumors in immunocompromised as a predictive system for testing chemotherapeutic agents, such as 5FU, and in determining optimal dosing schedules and combinations with other anticancer agents. As such, it is evident that the results from the present study cannot be utilized to support nor contradict our current knowledge of FA supplementation on chemosensitivity to 5FU. Future studies using Matrigel will have to be carefully tuned to provide the appropriate dosage of drug with respect to the xenograft burden.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSION AND FUTURE DIRECTIONS

6.1. General discussion

'Nutritional hormesis' is a term coined by D.P. Hayes in 2007, describing the biological and toxicological concept that small quantities can have opposite effects from large quantities, and relationships between nutrition and health outcome are not always dose dependent [381]. The hormesis model illustrates a U-shaped relationship between nutrient intake and adverse health outcomes. Thus, both nutrient deficiency and excess can have adverse effects to human health, and there is a level of intermediate intake which serves as the margin to maintain optimal health [382]. In a society where nutrient excess is now conceivable, it is important for health professionals and policy makers to be aware of the possible adverse effects.

In Canada, FA fortification was mandated in 1998, intended to provide an additional 100-200 μg/day to decrease the prevalence of NTDs. Folate plays an important role in nucleotide biosynthesis and one-carbon metabolism, critical processes in rapidly dividing cells. As such, adequate folate status is essential for expectant mothers, and fortification is unanimously a public health success among women of child-bearing age [2, 156-158]. However, it is unknown whether exposure at the population level can be considered with such high praise. Elevated blood folate concentrations have been implicated to mask vitamin B12 deficiency [383], decrease natural killer cell cytotoxicity [176], accelerate cognitive decline [177], and cardiovascular disease [246]. In addition, high folate status has been implicated in the risk and development of cancers of the prostate [184, 384], breast [187, 385] and colon (**Tables 2.6-2.11**), among others. The evidence looking at the relationship between high folate intake and CRC risk has been most studied, but remains controversial and conflicting. In general, large-scale epidemiological studies suggest an overall protective effect of folate at modest levels of supplementation [136-177]. By

contrast, preclinical and clinical studies suggest a possible harmful effect [220, 241, 263]. This inconsistency can be partially explained by the dual-modulatory role of folate on colorectal carcinogenesis [13, 106, 386]. In this paradigm, folate deficiency can predispose normal cells to undergo (pre)neoplastic transformation, due to the insufficient provision of substrates for normal cellular division to occur, whereas supplementation in this case can provide the necessary substrates ensuring the maintenance of DNA integrity [13, 106]. However, once preneoplastic lesions have been established, FA supplementation can further promote carcinogenesis [13, 106] and deficiency can suppress, and even inhibit tumor growth. Thus, the dose and timing of folate intervention is a critical factor to consider depending on the epithelial lining of the colon, and whether (pre)neoplastic transformation has occurred. Given that sporadic CRC has a latency period of 10-15 years, our aging population is likely to harbor preneoplastic lesions and be at most risk the harmful effects of FA supplementation. Over 70% of those taking supplements are over the age of 60 years, in fact 40% have detectable levels of UMFA after fasting [5, 167].

Furthermore, the ubiquitous and habitual use of dietary supplements, often containing a minimum of 400 µg FA, is seen among greater than half of the Canadian and American population [162]. Among cancer patients, nearly half report using a FA-containing supplement through the duration of their treatment [172, 173, 334]. Cancer patients often autonomously adopt supplement use, and do not consult their general practitioner or oncologist prior to use. In fact, a recent study suggests that supplement use is not necessary among the healthy population in developed countries such and Canada and the United States [387]. Furthermore the small percentage of individuals who had never taken a dietary supplement prior to diagnosis, are likely to adopt this new habit and continue throughout the course of their treatment.

5FU is an antimetabolite in which its primary mechanism is based on the interruption of folate-mediated *de novo* synthesis of nucleotides [17]. In addition, new *in vitro* evidence suggest that there are mechanisms of resistance that are induced by high levels of intracellular folate [19, 96, 319] that has important implications for cancer patients receiving 5FU-based chemotherapy. Particularly in its monoglutamated form, FA is readily taken up into enterocytes, unlike natural folates [1], it is imperative to understand whether higher intracellular levels can interact and affect chemosensitivity to 5FU-based chemotherapy. Thus, in a xenograft model of human colon carcinoma, we investigated the effect of FA supplementation on chemosensitivity to 5FU. Animals were randomized to receive 2, 8 or 25 mg FA/kg diet. Within diet groups, animals were further randomized to receive the Mayo Clinic regimen for non-metastatic CRC treatment or saline. Inhibition of xenograft growth served as a surrogate of response to chemotherapy and animals were euthanized at eight weeks post-treatment. Our data suggest that FA supplementation can decrease 5FU efficacy in a non-dose dependent manner.

6.2. Conclusion

In the present study, we sought to investigate whether FA supplementation can decrease chemosensitivity to 5FU in a xenograft model of human colon carcinoma. Though the mechanisms of folate-induced resistance were unclear, we found that FA supplementation can interfere and decrease chemosensitivity to 5FU in a non-dose dependent manner. This preclinical study adds to the growing body of evidence suggesting elevated pools of intracellular folate can modulate and interfere with chemotherapeutic drug metabolism. Given the drastically increased intake of folates, primarily from supplements, the present study warrants further studies to clarify the relationship between FA supplementation and chemosensitivity. Studies such as the present are thought-provoking and trigger questions of population-targeted public health initiatives and

self-directed healthcare. For the time being, it is advisable for the general population to consume no more than 400 µg of FA from supplements, unless otherwise advised by a physician, and aim to attain folates from natural sources. As well, cancer patients should not begin any health regimens without consulting their physician, as their self-directed behavior may in fact be compromising the effectiveness of their treatment.

6.3. Future directions

Data from the present study provides provocative evidence that FA supplementation may hinder response to chemotherapy, and future studies carried out in a larger scale in an effective xenograft model would confirm the findings of the present study (**Chapter 3)**. The addition of Matrigel proved to be too robust as demonstrated in the confirmation study (**Chapter 4)** and we were unable to see any effect of treatment on tumor growth, suggesting that the dosage of drug was no longer appropriate for the increase in tumor burden. Future studies must reconsider dosage in relevance to tumor burden, not only animal body weight or surface area, and would provide the statistical power necessary to draw more definitive conclusions.

As mentioned, HCT116 is an MSI-positive cell line, a CRC phenotype which has shown to be the least responsive to 5FU. It would be interesting to elucidate whether FA supplementation would greater affect chemosensitivity in a CIN-positive cell line, which represents the majority of sporadic cases, compared to a CIN-negative cell line. It is unknown why MSI-positive tumors respond more favorably to 5FU with the addition of platinum-based drugs compared to 5FU alone, but it has been postulated that the molecular events leading to CIN is more susceptible to interruption by anticancer agents, than a phenotype which has a defective MMR system, rendered early in carcinogenesis [58]. Replicating this study using HT29, a CIN-positive cell line, may provide a better understanding in how FA supplementation affects

response to 5FU, due to its favorable response to 5FU, compared to HCT116. CIMP may be more responsive to the effect of 5FU because of the promoter CpG island methylation of the genes implicated in CIMP may be highly susceptible to the effect of FA supplementation. However, CIMP cases vary in response because they can exhibit MSI- or CIN-positivity and as such, CIMP-positive are loosely characterized as greater than 100 CpG islands that have significantly higher levels of methylation, such as *CDKN2A, IGF2* and *MLH1 [388]*.

In the present study we were unable to elucidate mechanisms of resistance. Since MDR in response to 5FU has not been explored in human colon carcinoma prior to this study, it would be beneficial to investigate *in vitro* the relationship between HCT116 growth in folate-deplete, -replete and –supplemented media. *In vitro* experiments will provide the necessary foundation to formulate hypotheses for a subsequent *in vivo* study. There exists a lack of *in vivo* evidence to support recent *in vitro* observations of the role of the MDR proteins in response to chemotherapy.

Furthermore, the harvested tumor tissue was extremely heterogeneous presenting solid tumor, lymph fluid, necrotic tissue and blood. It may be advisable to shorten the duration of the study following the treatment period, such that the cancer does not display the invasiveness seen in this study. As such, the data for MDR expression analysis and immunohistochemistry may not be as variable. In the confirmation study, the duration of the study was shortened by three weeks for the reason that the animals no longer demonstrated tumor-inhibiting effects after five weeks. In addition, tumors of some animals surpassed 5000 mm^3 without compromising normal health and behavior; however we determined this as unnecessary tumor burden. Xenograft growth of the confirmation study was not responsive to 5FU at all levels of diet, rendering the data impractical for further analyses. Thus, a future study may benefit from terminating the study five

weeks following drug administration to elucidate mechanisms of active MDR without the cancer becoming too invasive and compromising rodent health beyond what is approved as per protocol.

Furthermore, it is necessary to clarify whether all metabolites of folate will behave in the same manner as FA. Studies have shown that there are critical differences in the uptake and metabolism between 5-methylTHF, FA and LV [389]. LV contributes directly to the intracellular pool of 5,10-methyleneTHF providing stability to the inhibition of the TS ternary complex, and decreasing treatment-related toxicity. A recent study suggests using 5-methylTHF with antifolate chemotherapy because it does not need to compete with natural folates for DHFR-mediated metabolism [389], which may in fact prevent treatment-related toxicities. Elucidating the biological differences of these folates will have important implications for cancer patients using dietary supplements while undergoing chemotherapy.

This preclinical study presents provocative findings which challenge the ubiquitous, yet unfounded, notion among the population that dietary supplement use is beneficial and necessary to maintain optimal health. In addition to validating the results with a larger sample size, an observational study in a clinical setting would provide crucial information on whether high blood folate concentrations can affect chemosensitivity to 5FU. Eligible CRC patients would provide blood samples, dietary information, and supplement use, in addition to consented access to treatment regimen. Although the collected data would reflect only the postfortification era, it would be interesting to observe whether high intakes of FA, primarily via supplement use, affects response to 5FU, compared to individuals who do not take supplements. To our knowledge, blood folate concentrations and supplement use of CRC patients have not been correlated to 5FU chemosensitivity. Thus, this clinical investigation would provide novel benchmarks of folate status in CRC patients in relation to their progress to become cancer-free.

Results of the present study suggest for the first time *in vivo* that FA supplementation may decrease chemosensitivity to 5FU. Though obvious metabolic differences between rodents and humans cannot be dismissed, our data suggest that an intermediate level of dietary FA, relevant to human consumption may interfere with chemotherapeutic efficacy. The findings from the present study in addition to emerging evidence from *in vitro* studies suggesting that FA can induce MDR, warrants the immediate need to elucidate the harmful effects of FA supplementation.

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