The Effect of Folic Acid and Methionine Deficiency and Excess on DNA Damage and Cancer Growth in HT29 Colon Cancer Cells and the Apc Min Mouse Model

A thesis submitted to the University of Adelaide
for the degree of Doctor of Philosophy

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ABSTRACT

Folate and methionine are critical for one-carbon metabolism, impacting DNA synthesis, repair, and methylation processes, as well as polyamine synthesis. These micronutrients have been implicated in colorectal cancer risk. The aim of this thesis was to examine in greater detail the role of folate and methionine in colon cancer initiation and progression by assessing DNA stability and tumour incidence. Studies were performed *in vitro* (using human colorectal adenocarcinoma HT29 cell line) and *in vivo* (using $Apc^{Min/+}$ mouse model).

The *in vitro* studies examining the effects of various folic acid and methionine concentrations within the physiological range on cell proliferation and genomic instability of HT29 cells, showed that restriction of folic acid or methionine inhibited cellular proliferation, while supra-physiological folate induced apoptosis. HT29 cells may be resistant to genome instability induced by folic acid or methionine deficiency under the experimental conditions reported for this study because no significant increases in micronuclei, nuclear buds or nucleoplasmic bridges were observed in the Cytokinesis-block micronucleus cytome (CBMN-Cyt) assay. The investigation on the effect of folic acid and methionine depletion on telomere length and DNA methylation in HT29 cells demonstrated that folate and methionine depletion may increase both telomere length and DNA methylation in HT29 cells. The length of telomere was positively correlated with DNA methylation.

In the *in vivo* studies using the $Apc^{Min/+}$ mouse model, the effect of supplementing a western-style diet with dietary folic acid and/or methionine on intestinal tumour development was assessed. A total of 113 mice were randomised to receive one of the four diet treatments; New Western Diet (NWD) as control diet, NWD with additional folic acid, NWD with additional methionine, and NWD with additional folic acid and methionine, administered at age of 3 until 13 weeks, with wild type (WT) mice used as controls. Supplementation of folic acid and methionine separately, resulted in marginally lower tumour numbers, when compared to the control diet. However, supplementation with both folic acid and methionine together appeared to annul the

marginal protective effect of supplementing individually. The investigation on the effect of supplementing a western-style diet with dietary folic acid and/or methionine on genomic stability (measured via micronucleated erythrocyte assay on blood sample; telomere length and DNA methylation on the colon tissue) showed insufficient evidence that additional folic acid and/or methionine promotes DNA stability or instability in $Apc^{Min/+}$ or WT mice. Dietary supplementation with folic acid and/or methionine at the levels and duration used in this study did not substantially promote or protect against DNA damage in WT or intestinal cancer-prone $Apc^{Min/+}$ mouse model fed a western-style diet although a marginal effect on tumour number was evident.

In conclusion, the results of this thesis support a role of methionine and folate in affecting intestinal cell proliferation and possibly tumour number. However, the impact of supplementation with folate and methionine on genome stability was marginal.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ARNIDA HANI TEH

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PRESENTATION AND PUBLICATION ARISING FROM THE THESIS

Abstract/Poster Presentation

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Publication

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LIST OF ABBREVIATIONS

5,10-MeTHF	5,10- methylenetetrahydrofolate
5-MeTHF	5-methyltetrahydrofolate
ACF	aberrant crypt foci
AHT	Arnida Hani Teh
ALT	alternative lengthening of telomeres
ANOVA	analysis of variance
AOM	azoxymethane
Apc	adenomatous polyposis coli
BER	base excision repair
BHMT	betaine:homocysteine methyltransferase
BN	binucleated
CB	Caroline Bull
CBMN Cyt assay	Cytokinesis Block Micronucleus Cytome assay
Cq	cycle threshold
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Cyto-B	cytochalasin-B
dcSAM	decarboxylated SAM
DFMO	α-difl uoromethylornithine
DHF	dihydrofolate
DMG	dimethylglycine
DNA	deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1

DSH	dishevelled
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
FAD	flavin adenine dinucleotide
FAP	familial adenomatous polyposis
FDA	Food and Drug Administration
FDR	false discovery rate
Folbp1	folate binding protein
GSK 3	glycogen synthase kinase 3
LRP	LDL receptor related protein
MF	Michael Fenech
MMRs	mismatch repair enzymes
MNi	micronuclei
MN-NCE	micronucleated normochromatic or non polychromatic erythrocytes
MN-PCE	micronucleated polychromatic erythrocytes
MTAP	methylthioadenosine phosphorylase
Mthdf1	methylenetetrahydrofolate dehydrogenase
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTSI	mucosal tissue of the small intestine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBUDs	nuclear buds

NCE	normochromatic or non polychromatic erythrocytes
NDI	nuclear division index
NPBs	nucleoplasmic bridges
NWD	New Western Diet
NWD+FA	New Western Diet with additional folic acid
NWD+FA+M	New Western Diet with additional folate and methionine
NWD+M	New Western Diet with additional methionine
OD	optical density
PBS	phosphate-buffered saline
PCE	polychromatic erythrocytes
qPCR	Quantitative Real-time Polymerase Chain Reaction
Rfc1	reduced folate carrier 1
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAMDC	S-adenosyl methionine decarboxylase
SCG	single copy gene
SD	standard deviation
SE	standard error
SHMT1	cytoplasmic serine hydroxymethyltransferase
SHMT1	serine hydroxymethyltransferase
TCF	T-cell transcription factor
THF	tetrahydrofolate

TS	thymidylate synthase
USA	United States of America
WT	wild type

Chapter 1:

General Introduction

1.1 Colorectal Cancer

Colorectal cancer is classically described as cancer that occurs in the colon, in a sequence of polyp-adenoma-carcinoma developing slowly over many years [1]. Colorectal cancer is reported as the second most common cancer in men and women in Australia (15 and 14%, respectively) [2] with 15,151 cases (in 2011) [3] and the incidence rates are higher in older populations (Figure 1.1) [4].

Previous evidence and trends have shown that the rates and incidence of this type of cancer is positively correlated with industrialisation, modernisation and urbanisation aspects of a country in general [5]. Nevertheless, various other factors including chemical carcinogens, mutated tumour suppressor genes, or spontaneous transformation have been linked with the development of cancer [6]. Stages of cancer development, which are initiation, promotion and progression, are modulated by several factors that are linked to metabolism, diet, and the environment. Free radicals (generated from oxidative stress) may cause various types of molecular damage to accumulate in cells including alterations to deoxyribonucleic acid (DNA), enzymatic activity, and induction of antioxidant and DNA repair protection mechanisms, thus influencing the development of cancer and its pathogenesis [7].

Cellular proliferation and apoptosis play important roles in tumour development. Generally, tumour growth is characterized by imbalance of cell proliferation and apoptosis (increased proliferation, decreased apoptosis, or both) [8, 9]. The first stage in tumour development which is tumour initiation is a result of mutation of epigenetic silencing of tumour suppressor genes (e.g. p53) causing an increase cell proliferation, beyond the capacity of normal cell [9, 10]. Normal cells undergo programmed cell death or apoptosis, while many cancer cells are resistance to apoptosis. The failure of cancer cells to undergo apoptosis contributes significantly to tumour development [10]. The abnormalities in cancer cells usually involves mutations in genes that regulate cell growth and differentiation [11].

The mutations of these genes are related to genetic and epigenetic instability in tumour development [9, 12-14]. The underlying mechanism include mutations, deletions, duplications, translocations, amplification, chromosome aberrations, viral infections and epigenetic inactivation [12, 14]. The genetic and epigenetic stability can be affected by extrinsic factors such as drugs [15, 16], dietary carcinogen [17], chemical carcinogen, radiation [10], and oxidative stress [18, 19].

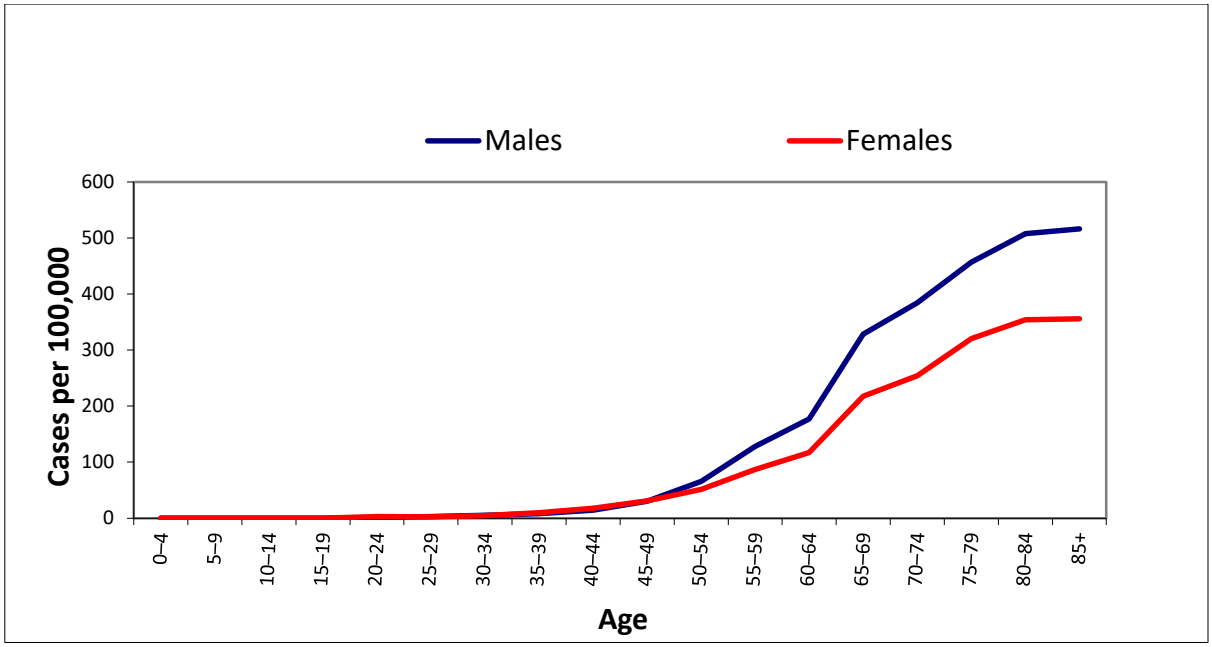

Figure 1.1: Age-sex specific incidence rates for colorectal/bowel cancer in Australia in year 2007 [4].

Association between dietary practice and cancer has been addressed and investigated thoroughly by researchers for many decades. The classical landmark review by Doll and Peto (1981) concluded that diet, together with other environmental contributing factors potentially cause cancers in human [20]. This assessment has led to encourage more studies to further investigate the relationship between dietary factors and cancer; in different aspects, perspectives and approaches. At the present time, the fact that there is relation between diet and cancer is becoming clearer and generally accepted. Since the association between food and cancer is extremely complicated, further studies are needed to provide the explanations and logical arguments to identify which aspects of this hypothesis are likely to be plausible.

Folate and methionine are amongst the micronutrients suspected of having a role in determining colorectal cancer risk [21-27], but there are discrepancies within the literature with some studies showing restrictions of folate and/or methionine to have tumour inhibitory effects, and others suggesting excess folate and/or methionine to have benefits. A cohort study from the Iowa Women's Health Study data showed no significant association found between folate or methionine intake and colorectal cancer incidence [28]. Meanwhile later, a meta-analysis of 7 cohort and 9 case-control studies that examined the association between folate consumption and colorectal cancer risk showed that folate has protective effect against colorectal cancer, with 25% lower risk among individual in the highest category of dietary folate intake compared to the lowest category [29].

In rodents, the combination of low folate and low methionine diet in rats resulted in the progressive hypomethylation of DNA in the liver, in association with altered one-carbon metabolism [30]. Meanwhile, studies in azoxymethane (AOM) treated rats showed that methionine restriction inhibits intestinal carcinogenesis [21, 31]. Interestingly, a study by Guruswamy et al. [32] demonstrated that supplementation of S-adenosyl methionine (SAM) inhibits colonic aberrant crypt foci (ACF) in AOM treated rats.

Folate and methionine may have protective effects as these two nutrients play important roles in DNA synthesis, repair and methylation. Folate is an important factor in one carbon metabolism, due to its requirement for genome stability. This is particularly because of the role of folate in supplying methyl groups for the synthesis process of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP), and as well as for the *de novo* synthesis of methionine from homocysteine; methionine is required for SAM synthesis [33]. Decarboxylated SAM synthesizes spermidine in the presence of spermidine synthase, and the reaction of spermidine with SAM synthesizes spermine catalysed by spermine synthase. In the methionine cycle, polyamine is produced which constitute a group of cell components that are important in the regulation of cell differentiation and proliferation [34]. DNA synthesis and repair, and DNA methylation processes are influenced by the presence of folate and methionine in the pathway.

In the United States of America (USA), the US Food and Drug Administration (FDA) authorized the addition of folic acid to grain products in March 1996, with compliance

mandatory by January 1998 [35]. In Australia, implementation of voluntary folic acid fortification of specified foods started in Western Australia in 1995 [36]. In 2007, the mandatory folic acid fortification standard was accepted by the authorities and is now being implemented nationally since 2009. The Australian standard requires all wheat flour for making bread be fortified with folic acid [37]. It is therefore essential to establish the effect that increased folic acid has on DNA stability and tumour development. These effects need to be investigated against a background of different methionine intake which varies in the Australian population [38].

1.2 Genetic Factors Affecting Colorectal Cancer

Apart from dietary, genetic factors also play an important role in affecting colorectal cancer. Mutations in several genes have been recognized to contribute to hereditary colorectal cancer [39]; mainly adenomatous polyposis coli (Apc), DCC (SMAD2/4), Src, MSH2, MSH6 MLH1 and other mismatch repair enzymes (MMRs), TP53 (p53), KRAS, BRAF and PTEN [40]. Mutation of tumour suppressor Apc gene is a strong risk factor, with frequency ranging from 70 to 80%, and is associated with the occurrence of familial adenomatous polyposis (FAP) and sporadic colorectal cancer [40].

Chapter 2:

Review: The Influence of Folate and Methionine on Intestinal Tumour Development in the $Apc^{Min/+}$ Mouse Model

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Contribution to the Paper	Identified, accessed and evaluated related references. Wrote the manuscript/review.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 26.4.2016

Name of Co-Author	Michael Fenech
Contribution to the Paper	Identified, accessed and evaluated related references. He critically reviewed the drafts and contributed to the writing of the paper. Acted as the corresponding author.
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Name of Co-Author	Caroline Bull
Contribution to the Paper	Identified, accessed and evaluated related references. She critically reviewed the drafts, edited the text and contributed to the writing of the paper.
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Name of Co-Author	Erin Symonds		
Contribution to the Paper	Critically reviewed drafts of the paper and contributed to the writing of the paper.		
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Contribution to the Paper	Critically reviewed drafts of the paper and contributed to the writing of the paper.		
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2.1 Abstract

Folate and methionine are critical for one-carbon metabolism impacting DNA synthesis, repair, and methylation processes, as well as polyamine synthesis. These micronutrients have been implicated in colorectal cancer risk. There are, however, inconsistencies within the literature, with some studies showing restriction to have tumour-inhibitory effects, whereas others suggest excess to have adverse outcomes. A review of the published data was conducted to examine the accumulated evidence for involvement of dietary folate and/or methionine restriction or excess in intestinal tumour development in the $Apc^{Min/+}$ mouse model, which is genetically prone to develop such cancers. Thirteen publications were identified and selected for evaluation based on the following inclusion criteria: (i) use of $Apc^{Min/+}$ mouse model; (ii) interventions using dietary folate and/or methionine; and (iii) primary outcome measures focused on intestinal tumour development. We found that nutritional modulation of folate and methionine was shown to have different effects on intestinal cancer in the $Apc^{Min/+}$ mouse, depending on the dosage, duration and timing of intervention, and interaction of the $Apc^{Min/+}$ genotype with other genetic factors affecting folate and DNA methylation metabolism. Although some studies showed that folate deficiency before tumorigenesis tended to increase risk of tumour formation, there are inconsistencies regarding whether excess folate post-weaning or after tumour initiation increases intestinal tumour burden. Altogether, the pooled data do not appear to indicate a difference in effect on intestinal tumour incidence between post-weaning diets that are folate deficient or folate adequate. The $Apc^{Min/+}$ mouse is a useful model for assessment of the impact of dietary folate on intestinal tumour

development, but further research is required to understand the reasons for these inconsistencies amongst studies based on likely mechanisms, including modulation of nucleotide synthesis, DNA methylation, and chromosomal instability, which may affect the rate of cellular division and its control.

2.2 Introduction

2.2.1 Background

Folate is a water-soluble vitamin that is essential for key cellular metabolic processes, and it occurs naturally in foods such as cereals and pulses [41] and green leafy vegetables [42]. In many countries, including the USA and Australia, the practice of mandatory folic acid fortification in wheat flour has provided an extensive range of food options that contribute to an individual's ability to meet the recommended daily requirement, which is 400 μg/day [43]. Adequate intake of folate can reduce the risk of neural tube defects, anaemia, vascular diseases, adverse pregnancy outcomes, neuropsychiatric disorders (as reviewed in [44]), atherosclerosis (as reviewed in [44, 45]) and colon cancer occurrence [46, 47]. Folate is essential for maintenance of genome stability due to its role as a methyl group donor for the synthesis of dTMP from dUMP, as well as for the *de novo* synthesis of methionine from homocysteine (refer to Figure 2.1) [48]. Reduced availability of methyl groups arising from folate deficiency leads to an increase in the dUMP:dTTP (deoxythymidine triphosphate) ratio, resulting in increased uracil incorporation into DNA. Extensive uracil excision by base excision repair (BER)

processes when folate is deficient can cause DNA strand breaks and chromosome instability [49].

A micronutrient that is metabolically related to folate is methionine. This essential amino acid is one of the key building blocks of protein, and it also has significant antioxidant properties both in its sulphur form [50] or as selenomethionine [50, 51]. Good dietary sources of methionine include Brazil nuts [52], flaxseed [53] and meat [54]. Methionine is essential in the methylation process as the precursor of SAM, a primary methyl-group donor (as shown in Figure 2.1) [55]. It is also required for cell growth as a precursor for the aminopropyl moieties of spermidine and spermine, the key polyamines required for transcription and translation, and which stimulate normal and neoplastic cell division [56, 57]. Current evidence suggests that dysfunction of the methionine cycle and/or methionine deficiency may be a contributing factor in the development of several types of cancer, including those of the liver and colon [58, 59].

Figure 2.1 shows a simplified diagram of the metabolic relationship between folate and methionine, under normal conditions, and the pathways by which these two nutrients interrelate in the synthesis, repair and methylation of DNA, and in the synthesis of polyamines.

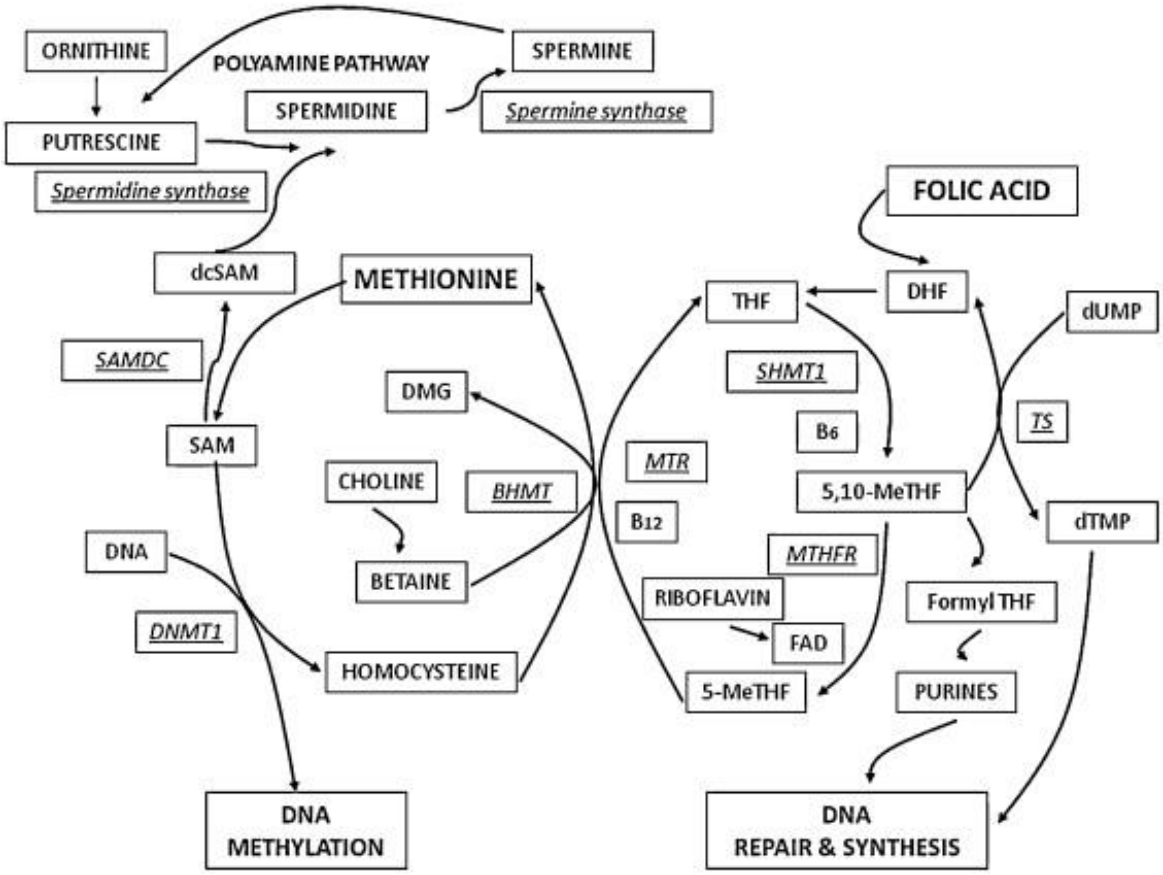


Figure 2.1: The main metabolic pathways by which folate and methionine interact in the process of DNA repair, synthesis and methylation, as well as synthesis of polyamines. Abbreviations: B6, pyridoxine; B12, cobalamin; BHMT, betaine:homocysteine methyltransferase; DHF, dihydrofolate; DMG, dimethylglycine; FAD, flavin adenine dinucleotide; 5-MeTHF, 5-methyltetrahydrofolate; 5,10-MeTHF, 5,10-methylenetetrahydrofolate; MTR, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; DNMT1, DNA (cytosine-5-)-methyltransferase 1; SAM, S-adenosyl methionine; SHMT1, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase; SAMDC, S-adenosyl methionine decarboxylase; dcSAM, decarboxylated SAM. Enzymes are underlined. Adapted from [48, 57].

Control of gene expression is effected through epigenetic processes such as methylation of cytosine residues in the DNA sequence of gene promoters. This process of DNA methylation is regulated by the ratio of SAM and S-adenosyl homocysteine (SAH) and catalysed by DNA methyltranferase enzymes, which transfer methyl groups from SAM to cytosines [60, 61]. Methionine and folate interrelate in this metabolic process at the stage of conversion of homocysteine to methionine. Dysregulation of DNA methylation has been consistently associated with cancer initiation (as reviewed in [62-64]). Chemical genotoxins, which may cause mutated and/or epigenetically silenced tumour suppressor genes, are amongst the main initiators of neoplastic changes [6]. The key stages of cancer development (initiation, promotion and progression) are modulated by factors which in turn can be linked to metabolism, diet [65, 66], and the environment (as reviewed in [67]). Since the landmark review by Doll and Peto [20], which was then proved by Boffetta [68], it has been concluded that diet constitute a means of protecting against or promoting cancer. Furthermore, numerous *in vivo* (human and animal) studies have been conducted to elucidate the mechanistic link between dietary practice and tumour development (as reviewed in [69, 70]).

In general, most epidemiological and experimental studies have specifically linked deficiency of dietary methyl donors, folate and methionine, with colon cancer risk (as reviewed in [22, 71]. It was hypothesised that this is due to imbalance in DNA methylation, in concert with compromised DNA synthesis and repair processes [72]. There are discrepancies, however, within the literature. Animal based studies propose that

insufficiency of folate might inhibit colorectal carcinogenesis, and excessive folate status may promote colorectal carcinogenesis [22, 26, 73]. Although human epidemiological studies tend to show an inverse relationship between folate status and the risk of developing colorectal cancer there are still concerns that an excessive folate intake may stimulate growth of initiated cancers [21, 74]. These observations in epidemiological studies merely propose the association and do not establish a cause and effect relationship between folate intake levels and the risk of colorectal cancer [22]. Folate may have a dual and contrasting effect on cancer, by preventing cancer-initiating chromosomal and epigenetic instability events and potentially stimulating the progression and growth of cancer after initiation (as reviewed in [75]). It is also becoming evident that genetic variation can have a significant bearing on folate transport and metabolism and the interaction of folate intake with genetic variation in folate transport and metabolism genes can influence the observed outcomes of experimental and molecular epidemiological studies of cancer and other diseases [73].

Meanwhile, the scarce data on the relationship between methionine and colon cancer, suggest that methionine may affect the development of colon cancer [46, 74]. Methionine restriction inhibits the development of colonic tumours in the colon of rats treated with AOM, mainly in post-initiation stage of carcinogenesis [21], whilst methionine addition was found to increase the surface area of tumours in the small intestine in the $APC^{Min/+}$ mouse [24].

2.3 The $Apc^{Min/+}$ Mouse Model of Intestinal Cancer

The $Apc^{Min/+}$ mouse is an important model for investigating intestinal cancer due to the presence of a genetic mutation in the tumour suppressor gene, Apc. This gene mutation is homologous to human APC germ-line mutations which cause the hereditary condition known as FAP [76]. An individual with FAP has an elevated risk of developing adenomatous colon polyps [77]. The number of adenomas formed can range from hundreds to thousands, many of which are anticipated to develop into colorectal cancer if left untreated [78]. FAP, attenuated FAP (where there are less than 100 polyps in the colon), and Gardner's syndrome (FAP with osteomas of the skull, epidermoid cysts, dental anomalies, and/or desmoid tumours) all result from germline mutations in the APC gene [79]. APC mutations have also been shown to contribute to the initiation of a majority of sporadic colorectal cancers [80-82], with inactivated APC found in 80% of sporadic colorectal cancer cases [83].

$Apc^{Min/+}$ mice on the C57BL/6 background display multiple intestinal neoplasia (Min) due to the mutant allele encoding a heterozygous mutation at codon 850 of the APC gene [84, 85]. As this mutation is very similar to that which causes FAP in humans it represents an ideal model to examine intestinal cancer development, as well as to test potential therapeutics. Similar to humans with APC gene mutation, $Apc^{Min/+}$ mice are predisposed to intestinal adenomas, developing several small intestinal adenomas and a few colorectal adenomas by age 60–120 days of age [85]. Whilst humans with FAP are most likely to develop tumours in the colon, 20–100% of FAP patients also develop tumours in the small

intestine (as reviewed in [86]). Unlike humans, however, tumours in the mouse model are more likely to occur in the small intestine [87].

APC is a large multidomain protein, consisting of 2843 amino acids, with several cellular roles including regulation of the WNT signal transduction pathway, cellular adhesion, migration, mitosis and apoptosis [88]. In the WNT signalling pathway (Figure 2.2), the APC protein serves as a negative regulator of intracellular levels of β-catenin, by promoting destruction of the latter [89]. In the absence of WNT signal (Figure 2.2a), β-catenin is degraded through its interactions with protein Axin, APC, and glycogen synthase kinase 3 (GSK3). In the presence of WNT signal (Figure 2.2b), the WNT proteins bind to a transmembrane protein (LRP) receptor. Thus, a signal is transduced to dishevelled (DSH) and Axin, leading to inhibition of the β-catenin degradation [90]. Stabilised β-catenin can then accumulate and enters the nucleus where it interacts with T-cell transcription factor (TCF), forming a heterodimeric transcriptional factor complex, which promotes the transcription of WNT responsive genes such as c-MYC, cyclin D2 and CD44 leading to cellular hyperproliferation [91, 92]. Excessive nuclear accumulation of β-catenin, can lead to developmental abnormalities and tumourigenesis [81].

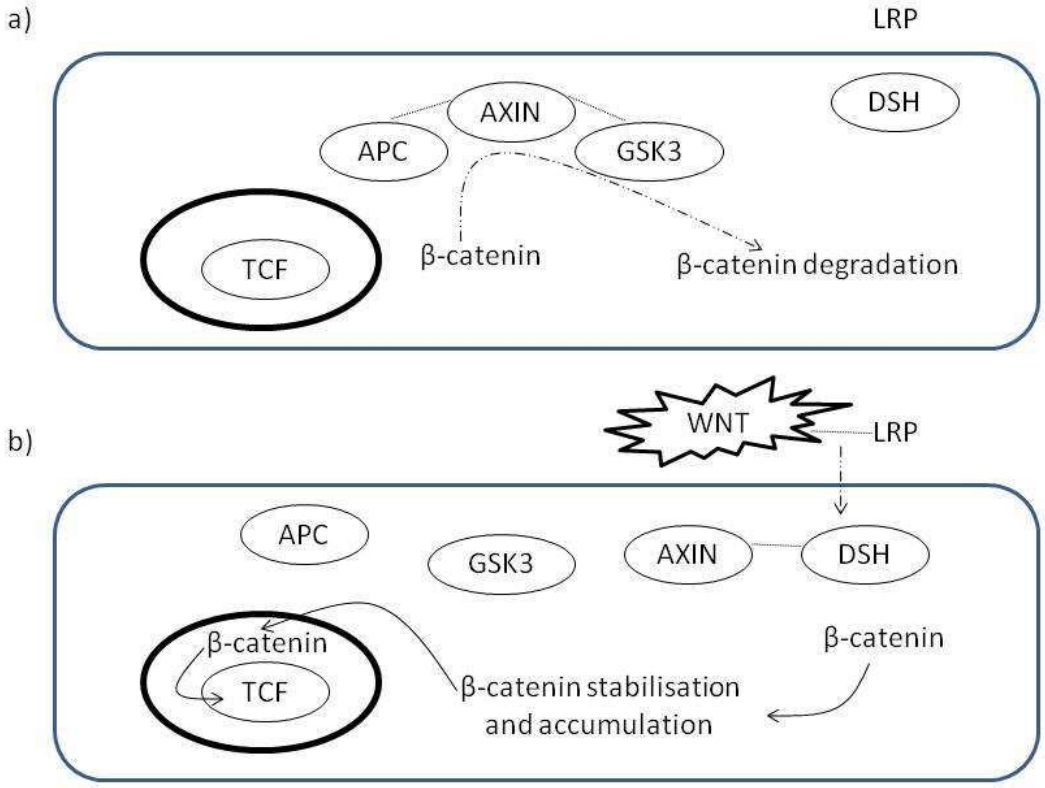


Figure 2.2: APC and WNT signalling pathway. (a) In the absence of WNT signal, β-catenin is degraded through its interactions with the proteins Axin, APC, and GSK3; (b) in the presence of WNT signal, the WNT proteins bind to LRP. Thus, a signal is transduced to DSH and Axin, leading to inhibition of the β-catenin degradation. Stabilised β-catenin can then accumulate and enters the nucleus where it interacts with T-cell transcription factor (TCF) causing stimulation of cell division.

Abbreviations: LRP, LDL receptor related protein; DSH, dishevelled; APC, adenomatous polyposis coli; GSK 3, glycogen synthase kinase 3; TCF, T-cell transcription factor.

Adapted from [90].

2.4 Literature Search and Inclusion Criteria

Herein we discuss the results of a thorough review of the current peer-reviewed literature on this topic, specifically including only those studies that met the following criteria: (1) use of $APC^{Min/+}$ mouse as the experimental model (this genotype has been notated as $Apc^{+/-}$, Apc^{Min}, and $Apc^{+/Min}$ in other literature); (2) interventions using dietary folate and/or methionine; and (3) primary outcome measures focused specifically on tumour development. Studies that also investigated effects on DNA methylation and genome stability parameters were also considered as this information is relevant for understanding mechanism of action. All references were accessed through publicly available electronic databases, and evaluated by three investigators include the PhD candidate (AHT), and her supervisors Dr. Caroline Bull (CB) and Prof. Michael Fenech (MF). The timescale employed starts from the first report describing the discovery of the Apc^{Min} mutation by Moser et al. (1990) [85] until before the review [93] being finalized for publication in 2012. The main reason the review article [93] is restricted to the APC^{Min} was because the thesis aimed specifically to understand how folic acid and methionine interact in affecting cancer with the mutation and furthermore the $APC^{Min/+}$ mice model was the model used in the *in vivo* study. The model is considered one of the better models to study the pathogenesis of a neoplasia in which the initial genetic defect is the same as that in the human.

The search used the following electronic databases: Medline, PubMed, Scopus, Web of Science. The reference lists for all obtained studies were hand-searched for additional

studies. All potential studies identified were independently evaluated for inclusion by two primary reviewers (AHT and MF). The primary reviewers were not blinded to the authors, institutions or source of publication at any time during the selection process. Disagreements about the inclusion/exclusion of studies were discussed and consensus achieved. Provision was made for a third reviewer if consensus was unattainable but did not prove necessary.

2.5 Studies Meeting the Inclusion Criteria

A comprehensive search of published literature generated thirteen studies which met the inclusion criteria of using the $Apc^{Min/+}$ model as the experimental system to investigate the effects of dietary folate and methionine on intestinal tumour incidence. A summary of the 13 studies [24, 94-105] is presented in Table 2.1, with highlights of the significant outcomes from each. Figure 2.3 provides a diagrammatic representation of the various dietary intervention designs used in these studies. For the purpose of this review and based on the reviewed papers basal adequate requirements of folate and methionine for rodents were considered to be 2 mg of folate and 4.6 g of methionine/kg diet respectively [106, 107]. Lesser content was considered deficient, and greater content considered as excess. Deficient diets were either 0 mg folate/kg diet or 0.2–0.4 mg/kg diet with sulphathiazole to suppress growth of folate-producing bacteria in the gut. Diets containing 8 mg or 20 mg folate/kg diet were considered to be excessive for this vitamin.

Table 2.1: Summary of published studies using the $Apc^{Min/+}$ mouse model to examine the effect of folate and/or methionine on tumour development and genome stability in chronological order of publication

Reference and description	Genotype	Sample size (n per group)	Dietary intervention	Start time of intervention	Duration of intervention (weeks)	Important outcomes
Song et al. [94] Investigate effects of folate	$Apc^{Min(+/-)}$	79 (8-11)	0, 2.0, 8.0, 20.0 mg folate/kg diet	Post weaning (3 weeks of age)	12-13, 25-26	Increasing folate decreased number of ileal polyps. Increasing folate decreased number of colonic aberrant crypt foci.
Song et al. [95] Investigate effects of folate	$Apc^{Min(+/-)}Msh2^{(-/-)}$	31 (7-10)	0, 8.0 mg folate/kg diet	Post weaning (3 weeks, i.e. before establishment of neoplastic foci or 6 weeks of age, i.e. after establishment of neoplastic foci)	5, 8	High folate decreased number of adenomas in small intestine. Folate supplementation from 3 weeks reduced intestinal adenomas, colonic ACF, and colonic adenomas by 2.7-2.8 fold. Folate supplementation from 6 weeks increased small intestinal adenomas by 4.2 fold.
Paulsen and Alexander [24] Investigate methionine effects	$Apc^{Min(+/-)}$	16 (3-5)	4.6 g, 11.6 g methionine/kg diet	Post weaning (4 weeks of age)	4	Number of tumours unchanged between treatments. Excess methionine increased surface area of small instestinal tumours.
Tucker et al. [96] Investigate effects of 5-fluorouracil and folate	$Apc^{Min(+/-)}$	274(6-37)	0, 4.0, 6.0 mg folic acid/kg diet	Post weaning (4 weeks of age)	7,12	Increasing folate increased tumour number.

Reference and description	Genotype	Sample size (n per group)	Dietary intervention	Start time of intervention	Duration of intervention (weeks)	Important outcomes
Sibani et al. [97] Investigate SAM, SAH, DNA methylation and folate/choline deficiency effects	$Apc^{Min(+/-)}$	43 (6-10)	0, 2.0 mg folate/kg diet	Post weaning (3 weeks of age)	10	Folate/choline did not produce a consistent effect on tumour number. Folate/choline deficiency decreased SAM. Folate/choline deficiency increased global DNA hypomethylation. Positive correlation between SAM, SAH or DNA methylation with tumour multiplicity.
Trasler et al. [98] Investigate DNMT1 and folate deficiency effects	$Apc^{Min(+/-)}DNMT1^{(+/-)}$	77 (3-14)	0, 2.0 mg folate/kg diet	Post weaning (3 weeks of age)	10	Folate deficiency decreased tumour number in $Apc^{Min(+/-)}DNMT1^{(+/-}$ but not in $Apc^{Min(+/-)}DNMT1^{(+/+)}$ mice. DNMT1 deficiency with or without folate deficiency decreases tumour number in $Apc^{Min(+/-)}$ mice.
Bashir et al. [99] Investigate folate in the context of multiple vitamin deficiency or excess	$Apc^{Min(+/-)}$	180 (30)	0.3, 1.0, 2.0 mg folate/IU	Post weaning (3 weeks of age)	8	Reduced or increased folate conditions in parallel with corresponding multivitamin changes increased number of polyps and the tumour burden, crypt fission and weight of small intestine.
Lawrence et al. [100] Investigate interaction effect of Rfc1 knockout, MTR knockout and folate deficiency effects	$Apc^{Min(+/-)}Rfc1^{(+/-)}$, $Apc^{Min(+/-)}MTR^{(+/-)}$	79 (3-16)	0.2-0.3, 2.0 mg folic acid/kg diet	Post weaning (3 weeks of age)	7	Folate deficiency increased adenoma number, plasma homocysteine, apoptosis in all genetic backgrounds. Folate deficiency decreased global DNA methylation in all genetic backgrounds. Rfc1 deficiency reduced adenoma number and load, but MTR deficiency had no such effect.

Reference and description	Genotype	Sample size (n per group)	Dietary intervention	Start time of intervention	Duration of intervention (weeks)	Important outcomes
McKay et al. [101] Investigate in utero folate deficiency effects	$Apc^{Min(+/-)}$	148 (37)	0.26, 0.4, 2.0 mg folic acid/kg diet	In utero and after birth (intervention starts on breeding mice, throughout pregnancy and nursing)	10	No significant effect of folate dose on tumour number or size.
Lawrence et al. [102] Investigate MTHFR and folate effects	$Apc^{Min(+/-)}MTHFR^{(+/-)}$	59 (8-13) 70 (2-12)	0.3, 2.0, 20.0 mg folate/kg diet 0.3, 2.0, 20.0 mg folate/kg diet	Post weaning (3 weeks of age) offspring (dietary intervention started prior to mating, throughout pregnancy and nursing)	7+	Mice on high folate diet from weaning developed more adenomas. MTHFR had no impact. Offsping of mice on low folate diet and subsequently on the same diet post-weaning developed fewer adenomas. $MTHFR^{+/-}$ genotype associated with reduced adenoma numbers in the $Apc^{Min/+}$ offspring.
MacFarlene et al. [103] Investigate Mthfd1 and folate deficient effects	$Apc^{Min(+/-)}Mthfd1^{(+/-)}$	32 (8)	0, 2.0 mg folate/kg diet	Post weaning (3 weeks of age)	11	Folate deficiency and Mthdf1 had no effect on tumour in the $Apc^{Min/+}$ mice.
MacFarlene et al. [104] Investigate SHMT1 and folate effects	$Apc^{Min(+/-)}SHMT1^{(+/-)}$	NA (4-13)	0, 2.0 mg folate/kg diet	Post weaning (3 weeks of age)	11	Folate deficiency increased tumor number and load in $Apc^{Min/+}SHMT1^{-/+}$, but had no effect in $Apc^{Min/+}SHMT1^{+/+}$, or $SHMT1^{-/-}$ mice. Folate deficiency increased uracil content in $Apc^{Min/+}$ mice with SHMT1 knockout.
McKay et al. [105] Investigate maternal folate deficiency effects on DNA methylation in the offspring	$Apc^{Min(+/-)}$	NA (10-21)	0.4, 2.0 mg folate/kg diet	In utero and after birth (intervention starts on breeding mice, throughout pregnancy and nursing)	9+	Maternal folate deficiency reduced p53 methylation in adult mice. Folate deficiency post-weaning increased APC methylation in the $Apc^{Min/+}$ mice.

Abbreviations: SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; DNMT1, DNA (cytosine-5)-methyltransferase 1; Rfc1, reduced folate carrier 1; MTHFR, methylenetetrahydrofolate reductase; Mthdf1, methylenetetrahydrofolate dehydrogenase; SHMT1, cytoplasmic serine hydroxymethyltransferase.

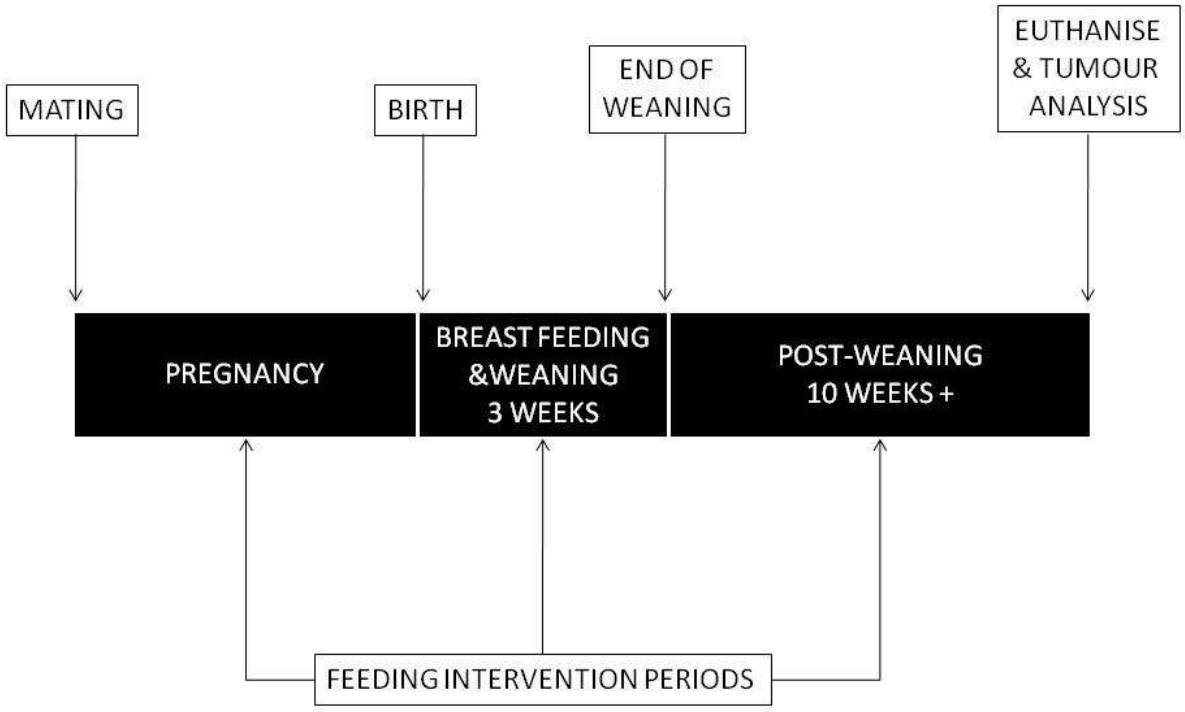


Figure 2.3: Summary of typical dietary intervention study designs used for folate and/or methionine interventions in $Apc^{Min/+}$ mouse studies. Intervention studies varied depending on when the dietary change intervention was commenced or finalized. The intervention periods investigated were either (i) during pregnancy, (ii) for the first 3 weeks after birth when baby mice are still being breast fed and/or weaned and (iii) post-weaning. Typically mice were sacrificed 10 weeks or later after weaning. Most of the studies performed interventions in the post-weaning period.

2.5.1 Studies Which Investigated Folate Deficiency Only

Seven studies investigated the effects of folate deficient diet (0 mg/kg diet) relative to folate adequate diet (2 mg/kg diet) in different scenarios of diet and other genetic factors. Sibani et al. investigated the effect of folate/choline deficiency, together with levels of SAH and SAM, and global DNA methylation in the small intestine in the $Apc^{Min/+}$ mice [97]. Forty-three mice (6–10 per group) were fed a diet deficient for both folate and choline (0 g of folate/kg diet), or a control diet containing 2 mg folate/kg diet at weaning. Mice were killed at 13 weeks of age. Positive linear correlations between SAH or SAM and number of tumours ($p < 0.005$), and between global DNA hypomethylation and tumour multiplicity ($p = 0.014$), were observed in mice receiving the diet containing folate [97]. From their results these researchers concluded that folate/choline deficiency did not produce a consistent effect on tumour number in replicate experiments, possibly because of other genetic differences between mice in the different experiments with regards to the transformation state of cells in the intestine and susceptibility to DNA hypomethylation.

Genomic methylation is mediated by DNA (cytosine-5)-methyltransferase 1 (DNMT1) [108]. Trasler et al. studied the effects of reduced DNMT1 expression together with adequate or low dietary folate, to explore the combination of genetic and dietary factors on global DNA methylation and small intestinal tumour development [98]. Promoter CpG island methylation profile of E-cadherin and p53 genes were also evaluated. To achieve the objectives, the $DNMT^{(+/-)}$ mutant $Apc^{Min/+}$ mouse model was used. Two experiments were carried out with 77 mice ($n = 29$ for experiment 1, and $n = 48$ for experiment 2,

with *n* = 3–14 per group) given either a folate deficient diet (0 mg of folate/kg diet) or a control diet (2 mg folate/kg diet) post weaning. Mice were sacrificed at 13 weeks. With regard to DNMT1, $Apc^{Min/+}$ mice with mutant DNMT1 had significantly fewer tumour numbers (by ~40 %) compared to $Apc^{Min/+}$ with normal DNMT1, in both the folate deficient and control group. Assessment of the dietary and genetic manipulation showed that folate deficiency in the $Apc^{Min/+}$ mice with mutant DNMT1 resulted in decreased tumour numbers (by ~70 %), compared to other groups. However, the hypothesis that low level of dietary folate could expedite tumorigenesis in the $Apc^{Min/+}$ was not proved. A 10-week (from weaning to 13 weeks of age) period of folate deficiency did not accelerate tumorigenesis in $ApcMin^{/+}$ mice (without DNMT1 mutation). Folate deficiency in this study resulted in decreased concentration of SAM, which is essential for DNA methylation. However, none of the mice in the folate deficient diet group showed changes in DNA methylation. In summary this study showed that folate deficiency was marginally associated with decreased tumour number, and did not accelerate tumorigenesis. Folate intake did not influence overall genomic DNA methylation or the methylation levels of E-cadherin or p53 in intestinal tissue, regardless of the presence or absence of functional DNMT1 [98].

A study by Lawrance et al. used a genetically modified $Apc^{Min/+}$ mouse, which was genetically deficient in cellular folate uptake or folate metabolism, to study the effects of folate deficiency on tumorigenesis [100]. To generate these double mutant strains the $Apc^{Min/+}$ mice were crossed with reduced folate carrier 1 (Rfc1) knockout mice or with methionine synthase (MTR) knockout mice. Rfc1 acts as transporter of 5-methylTHF into

cells whereas MTR is the enzyme required for transferring the methyl group from 5-methylTHF to homocysteine to produce methionine, which is then converted to SAM. Seventy-nine mice (3–16 per group) were assigned to receive either a control diet containing 2 mg folic acid/kg or a folic acid deficient diet containing 0.2–0.3 mg folic acid/kg. Mice were killed at 10 weeks of age. Results showed that folate deficiency increased the number of adenomas (by 49 %) in both $Mtr^{+/+}Apc^{Min/+}$ and $Mtr^{+/-}Apc^{Min/+}$ mice, although dietary folate appeared to have no significant influence on size of adenomas. The folate deficient diet caused a significant increase in plasma homocysteine in all genotypes ($Mtr^{+/+}Apc^{min/+}$, $Mtr^{+/-}Apc^{Min/+}$, $Rfc1^{+/+}Apc^{Min/+}$ and $Rfc1^{+/-}Apc^{Min/+}$), as well as an increase in caspase-3/7 activity suggesting an increased rate of apoptosis. It was also found that the $Apc^{Min/+}$ mice with knockout Rfc1 developed fewer adenomas, as well as reduced adenoma sizes [100]. From this study, it was concluded that genetic and nutritional alterations in folate bioavailability may impact tumorigenesis in $Apc^{Min/+}$ mice.

A study by McKay et al. hypothesised that insufficient folate *in utero* and during early life may affect tumorigenesis in the offspring [101]. Female C57Bl6/J mice were randomised to a folate adequate (2 mg folic acid/kg diet) or folate deficient (0.4 mg folic acid/kg) diet throughout mating (with $Apc^{Min/+}$ male), pregnancy and lactation. 148 $Apc^{Min/+}$ offspring (37 per group) were then randomly assigned to receive folate adequate (2 mg folic acid/kg diet) or folate deficient diet (0.26 mg folic acid/kg) for 10 weeks post-weaning. Results showed no evidence that maternal folate deficiency affected

tumorigenesis in the offspring. However, female offspring mice fed normal folic acid diet post-weaning had more (by 150 %) and larger tumours compared to folate-depleted females post-weaning.

Two recent studies by MacFarlane et al. [103] and [104], used genetic modification of methylenetetrahydrofolate dehydrogenase 1 (Mthfd1) and cytoplasmic serine hydroxymethyltransferase (SHMT1) to study effects of folate deficiency in the $Apc^{Min/+}$ mice. Both Mthfd1 and SHMT1 play an important role in folate metabolism, with Mthfd1 providing folate-activated-one-carbon units in the cytoplasm [103], whilst SHMT1 is required for the synthesis of 5,10-methylenetetrahydrofolate which is the form of folate essential for synthesis of dTTP from dUMP [104]. The first study [103] compared two models of intestinal cancer; (i) crosses between *Mthfd1*$^{gt/+}$ and $Apc^{Min/+}$ mice, and (ii) AOM (chemically)-induced colon cancer in *Mthfd1*$^{gt/+}$ mice. Post-weaning, mice were grouped in $n = 8$ per group, and randomly assigned onto control diet containing 2 mg folic acid/kg diet or folate deficient diet containing 0 mg folic acid/kg diet. Dietary interventions were maintained for 11 weeks. *Mthfd1* had no significant effect on tumour incidence in the $Apc^{Min/+}$ mice. However, a non-significant trend indicating a reduction in tumour number and size was recorded in groups fed the folate deficient diet which caused a decrease in folate concentration compared to the control group.

The second recent study by this group [104] looked at the interaction of SHMT1 gene and dietary folate in modifying susceptibility to intestinal cancer in the $Apc^{Min/+}$ mice.

Mice were fed with either a folate adequate control diet with 2 mg folic acid/kg diet or folate deficient diet containing 0 mg folic acid/kg diet. Mice were maintained on the dietary regimen from 3 to 14 weeks of age. Folate deficiency decreased tissue folate concentration, increased total tumour number (by 50 %) and load in *ApcMin*$^{/+}$*SHMT1*$^{-/+}$, but had no effect in *ApcMin*$^{/+}$*SHMT1*$^{+/+}$, or *SHMT1*$^{-/-}$ mice. It was also found that folate deficiency increased uracil content in hepatic nuclear DNA in Apc$^{Min/+}$ mice with SHMT1 knockout.

Recently, McKay et al. [105] published their investigation on effects of maternal folate deficiency on DNA methylation in the offspring. Female C57Bl6/J mice were given either a folate replete (2 mg folic acid/kg diet) or low folate (0.4 mg folic acid/kg diet) diet, from mating with Apc$^{Min/+}$ males, throughout pregnancy and lactation. Offspring (both Apc$^{Min/+}$ and wild type) were randomly assigned to receive low or high folate diets, post-weaning to an average of 96 days of age. Methylation of specific genes (pf3, Igf2, p16 and APC) were assessed at weaning and in the adult offspring. Overall, it was found that maternal folate depletion reduced p53 methylation in adult offspring. Folate deficiency post-weaning increased APC methylation in the Apc$^{Min/+}$ mice only.

2.5.2 Studies with Both Folate Deficiency and Excess

Four out of 13 studies were done with both deficient and excess levels of dietary folate and effects assessed relative to adequate levels of folate in the diet (i.e. 2 mg/kg diet; excess levels were typically 8 mg folate/kg diet or greater). The first by Song et al. was designed to investigate the effects of 0, 2, 8 and 20 mg/kg of folate in the diet on the

development of intestinal polyps in $Apc^{Min/+}$ mice [94]. Seventy-nine mice (8–11 per group) were fed with assigned diets from weaning, prior to sacrifice following either 3 or 6 months on the intervention. Adenomas in the small intestine and ACF, and adenomas in the colon were determined. Increasing folate was found to cause a significant reduction in the number (by 75-100 %) of ACF in the colon after 3 months. Whilst at 6 months, no significant differences in ACFs were observed amongst all groups. After 3 months of dietary intervention, however, the higher folate diets were shown to significantly reduce the number of adenomas in the ileum, with animals in the 20 mg/kg diet group showing a 68–78% reduction in the number of ileal adenomas compared with the three lower folate diets. After 6 months intervention this effect had reversed, with the group given the diet containing 0 mg of folate/kg displaying lower numbers (by 62-76 %) of ileal adenomas than the other three diets. In summary, results obtained from this study suggest that dietary folate supplementation reduced the development of ACF in the colon in the short term but not in the long term. The results for the development of adenomas in the ileum were conflicting and were dependent on duration of intervention. Serum folate concentration was lowest in the group fed with 0 mg of folate/kg, compared to other groups, at both 3 months and 6 months time point; however, the mean folate concentration within each group was not significantly different at 3 months and 6 months indicating that the changes in adenoma formation with time were not confounded by fluctuations in tissue folate concentration with time.

Another study by the same group was carried out using an $Apc^{Min/+}$ model with a null mutated *Msh2* gene ($Msh2^{-/-}$) designed to investigate the extent to which folate in the diet may affect the progress of developing intestinal tumorigenesis [95]. During cell division, *Msh2*, a mismatch repair gene is involved in maintaining fidelity of genome replication, and defects in this type of DNA repair gene are associated with increased risk of intestinal cancer in human [109]. Mice were randomly assigned to receive a diet containing either 0 or 8 mg of folate/kg starting from weaning at 3 weeks of age (before the development of neoplastic foci), or 3 weeks later (after the development of neoplastic foci). Mice were sacrificed at 11 weeks of age. As with the previous study from this group, ACFs in the colon and adenomas in the small intestine and colon were analysed. Folate supplementation starting at the end of weaning (at 3 weeks) was found to significantly reduce the number of adenomas in both small intestine (by 63 %) and colon (by 65 %). In contrast, folate deficient diet, which caused a drop in serum folate concentration, significantly reduced the number of adenomas in the small intestine (by 76 %) if commenced after the development of neoplastic foci (6 weeks). However there was no effect on colon ACFs and adenomas. Based on their analyses of global methylation, these researchers concluded that there was no significant relation between genomic DNA methylation and the development of tumorigenesis in this study, regardless of the duration and starting point of folate interventions. In summary the researchers concluded from this work that dietary folate supplementation before neoplastic foci formation attenuates the development of tumorigenesis in both the small intestine and colon. However, timing of the intervention was shown to be important because folate supplementation after

development of neoplastic foci (i.e. at 6 weeks of age) resulted in a 322 % increase in incidence of small intestinal adenomas.

Later, a study carried out by Tucker et al. investigated the response of 5-fluorouracil chemotheraphy with dietary folic acid (at 0, 4.0 and 6.0 mg/kg diet) in $Apc^{Min/+}$ model [96]. Mice (*n* = 6–37 per group) were assigned to received dietary regimens starting at 4 weeks of age, and tumour number were evaluated at 10 and 15 weeks of age. In general, their data suggest that mice on folate deficient or excess diet developed lesser tumour number (by 44-81 %), compared to mice fed with standard rodent chow diet (containing 2 mg folic acid/kg diet).

A more recent study by Lawrance et al. determined the combined effects of MTHFR genotype and folate deficiency on tumorigenesis in the $Apc^{Min/+}$ genetic background [102]. Diets with variable folate content (0.3, 2.0, 20.0 mg folic acid/kg diet) were administered either pre-natally or at weaning. Mice fed high folate diets from weaning developed more adenomas (by ~50-80 %) than those on low folic acid or deficient diets, but MTHFR genotype had no impact. Offspring of dams fed with folate deficient diet and subsequently on the same diet post-weaning developed fewer adenomas; $MTHFR^{+/-}$ genotype of the mother, or of the offspring, was also associated with reduced adenoma numbers (by ~45 %) in the $Apc^{Min/+}$ offspring. Adenoma number was inversely associated with plasma homocysteine and intestinal dUTP/dTTP ratio [102].

2.5.3 Study with Folate as a Multivitamin Component

There is a distinctive study conducted by Bashir et al. [99], designed to test the effects of altering the content of multiple vitamins in the $Apc^{Min/+}$ mouse model. One of the vitamin components that was altered in the diet was folate. 180 mice (30 per group) were used to examine the effects of modified vitamin levels on the development of intestinal polyps, cell proliferation and crypt fission (a physiologic mechanism of crypt reproduction). Modifications were made to the concentration of several vitamins and micronutrients in the diet (A, B1, B2, B3, B5, B6, and B12, folate, biotin, C, D, E, K and selenium). At the age of 4 weeks, mice were assigned either to the control diet (basal levels of vitamins and micronutrients, containing 1.0 mg folate/kg diet), low vitamin diet (depleted vitamin and micronutrient content, containing 0.33 mg folate/kg diet), or high vitamin diet (replete vitamin and micronutrient content, containing 2.0 mg folate/kg diet). Animals were killed at 12 weeks of age, after 8 weeks on the treatment diets. Polyps, cell proliferation and crypt fission were then evaluated. The diets containing both low and high folate resulted in an increase in polyp number in the small intestine (70-80 %), compared to controls. Both low and high folate supplementations (as part of the diet contents) showed a small decrease in cell proliferation. Only the diet containing high folate content had a significant effect on decreasing crypt fission. The authors acknowledged the possible role of folate in providing such effects [99], although interactions with other vitamin intake changes might be involved. This study is unique, and not strictly comparable to other studies mentioned earlier due to a few factors: (1) amount of folate was modified together with other vitamins which makes it impossible attribute the effects solely to folate; and (2) definition of high

and low folate differs from other studies, because 2.0 mg of folate is classified as high in this study whereas 2.0 mg of folate is considered as basal dosage in other studies discussed above.

2.5.4 Study with Methionine

To our knowledge only one study investigating the effects of dietary methionine supplementation in the development of intestinal adenomas in the $Apc^{Min/+}$ mouse model has been reported to date, by Paulsen and Alexander [24]. Sixteen mice (3–5 per group, mixed male and female) were assigned either to the standard AIN-76A diet (containing 4.6 g of methionine/1 kg diet) or the AIN-76A diet with added 0.7% L-methionine (equivalent to 11.6 g of methionine/1 kg diet) for 4 weeks, commencing from 4 weeks of age, prior to sacrifice at the age of 8 weeks. Results showed that the additional methionine promoted the growth of adenomas in the small intestine by increasing the surface area of tumours by 41%, whilst having no effect on the number of tumours in both the small intestine and the colon [24]. The very short duration of treatment in this study makes it difficult to compare with folate intervention studies which were all carried out for longer duration.

2.6 Proposed Mechanisms for the Effects of Folate and Methionine on Intestinal Tumour Growth in $Apc^{Min/+}$ Mice

The mechanisms by which dietary methionine and/or folate may affect intestinal carcinogenesis are speculative at this stage. There are at least three possibilities: (1) modulation of nucleotide synthesis caused by folate deficiency or excess; (2) DNA

methylation changes due to deficiency of methyl donors which may alter gene expression; and (3) altered polyamine metabolism which may affect the rate of cellular division.

2.6.1 Modulation of Nucleotide Synthesis Caused by Folate Deficiency or Excess

Folate (in the form of formyl THF) is essential for one carbon metabolism and involved in the conversion of dUMP to dTMP, and in the synthesis of purines [110].

Severe folate deficiency leads to an excess of dUMP and increased incorporation of uracil in DNA which is mutagenic and impairs DNA synthesis and repair [111-113]. Folate insufficiency is known to imitate clastogenic genotoxin exposure by inducing single and double strand breakage [114]. Mechanistically, this effect arises from a reduction in the pool of DNA precursors leading to DNA replication stress or, transient DNA breaks caused by excision repair of uracil from DNA thus inhibiting proliferation and DNA synthesis in multiplying adenoma cells [100]. As such it is proposed that folate deficiency may slow tumour growth by increasing DNA damage [100] and cell cycle delay by checkpoint mechanisms (as reviewed in [115]). The replication stress caused by impaired folate metabolism inhibits cell division which is the central principle of methotrexate and 5-fluorouracil, both anti-folate agents, used as antitumour therapies [116].

In the $Apc^{Min/+}$ mouse model the data is conflicting, with some studies showing folate having an inhibitory effect, whilst in other circumstances it is stimulatory of tumorigenesis. The effects appear to be dependent on the timing and dosage of the

intervention. For example, it has been suggested that dietary folate may suppress the development of polyps when implemented at an early stage (upon weaning), but may show an opposite effect when given at later stage [94, 95]. Genetic defects which alter folate transport proteins such as Rfc1 [117], could also affect the formation of adenoma in this model [98, 100]. In contrast, in mouse models which were genetically normal for APC but had folate binding protein (Folbp1) or Rfc1 genes ablated, treatment with the colon carcinogen AOM resulted in larger adenocarcinomas in Rfc1 deficient mice and more tumours occurred in the Folbp1 defective mice relative to their genetically normal counterparts [110]. These conflicting results suggest that folate might have opposing effects acting as an inhibitor at the initiation phase of carcinogenesis which is driven by mutagenesis, whilst acting as promoter in the latter stages by stimulating cellular proliferation [99]. However, because tumours may be initiated or promoted at different time-frames along the intestine, there is likely to be some overlap in their chronicity which creates uncertainties for determining the appropriate timing for folate interventions.

In the murine model, weaning offspring are dependent on maternal supply of folate (in the form of 5-MeTHF) from their mother's milk, and their own folate uptake mechanisms [87, 100, 118]. It has been shown that intestinal cells are more prone to folate-deficiency induced adenoma formation in the newborn, compared to adult mice [87]. This supports the significance of folate as an inhibitor at the initiation phase of carcinogenesis.

2.6.2 DNA Methylation Changes Due to Deficiency of Methyl Donors Which May Alter Gene Expression

One of the speculated mechanisms underlying the effect of dietary folate and/or methionine on intestinal carcinogenesis is through alterations in DNA methylation. Deficiency in these micronutrients results in reduced levels of SAM, altered DNA methylation patterns and enhanced mutation rates [119]. DNA methylation is inversely linked with gene expression, with methylation of CpG islands adjacent to promoters down-regulating gene transcription [120, 121]. Alterations in DNA methylation and levels of DNA methyltransferase expression have been associated with tumorigenesis [122, 123]; specifically global DNA hypomethylation has been reported in colonic neoplasia [124, 125]. Methyl group deficient diets lead to DNA hypomethylation in rats [126-128], and in humans [129]. In pre-neoplastic small intestinal cells, global DNA hypomethylation is positively correlated with tumour multiplicity in mice fed adequate folate [97]. These findings are in agreement with a previous study showing DNA hypomethylation in neoplastic cells (from human colon tissue) [130, 131]. Interestingly, Trasler et al. demonstrated that folate deficiency can decrease tumour number without influencing overall genomic DNA methylation status, suggesting that other mechanisms (e.g. uracil incorporation into DNA) are also implicated in tumorigenesis [98]. On the other hand, aberrant DNA methylation also affects the development of mutations by causing incorrect transcriptional control of tumour suppressor (e.g. silencing of DNA repair and cell cycle checkpoint genes) and tumour promoter genes (e.g. activation of parasitic oncogenic viral DNA) consequently increasing tumour risk [132].

2.6.3 Altered Polyamine Metabolism Which May Affect the Rate of Cellular Division

Intestinal epithelium may utilise methionine generated in the folate/methionine metabolic cycle (Figure 2.1) or dietary methionine to meet the requirement of polyamines for cellular hyperproliferation in tumorigenesis [56, 133]. There is an inter-related connection between polyamine synthesis, WNT signalling and the APC tumour suppressor gene in intestinal epithelium. APC down-regulates β-catenin. WNT signals modulate the stability of a protein complex (containing β-catenin). In the absence of WNT or the presence of wild-type APC protein, β-catenin is degraded by the 26 S proteasome. In contrast, in the presence of WNT, or the absence of APC, β-catenin target genes (including c-*myc)* are expressed. The expression of c-myc leads to the increased expression of ornithine decarboxylase (ODC), an enzyme involved in polyamine synthesis which leads to polyamine-induced increase in cell proliferation [134]. The synthesis of polyamines is ultimately dependent on methionine supply because decarboxylated SAM is the precursor molecule essential for polyamine synthesis [95]. Folate and methionine status possibly modulate the initiation of tumorigenesis through their role in polyamine production [133, 134]. Hence, the higher SAM observed in the pre-neoplastic small intestine of $Apc^{Min/+}$ mice may signify an increased potential for polyamine synthesis [97]. In contrast polyamine depletion was shown to reduce adenoma development in the $Apc^{Min/+}$ mouse model [135].

2.7 Knowledge Gaps and Conclusions

With the limited literature available, the mechanisms by which dietary methionine or folate may affect tumorigenesis in the $Apc^{Min/+}$ mouse model remain unclear and speculative. Although this model has been used extensively in investigating dietary effects of intestinal cancer the mice mainly develop small intestinal polyps in contrast to humans for whom intestinal cancer develops mainly in the colon [94]. In this review, we focussed on the role of methionine and folate, although it should be noted that there are other components of the mouse diets that may vary between studies such as protein, fat, carbohydrate and vitamin contents which could further modify susceptibility to carcinogenesis. As a result, several key questions still need to be addressed to clarify our understanding of the beneficial or detrimental effects of dietary folate and methionine on intestinal tumorigenesis in $Apc^{Min/+}$ mouse model such as:

- What are the optimum intakes of folate and methionine in ageing mice to prevent growth of intestinal cancer?
- When is the right time to implement folate and methionine restriction or supplementation to prevent or control cancer growth?
- How do different genetic and dietary backgrounds alter the effects of folate and methionine deficiency or excess on the conversion of a benign polyp to an adenoma?
- Is there an interactive effect of folate and methionine and which of the two has the strongest impact on intestinal tumour incidence and progression?

- What are the gene expression pattern changes that diagnose a trajectory to malignancy in this model?

Sex-specific effects of folate and/or methionine on tumour development, was rarely tested or discussed in the reviewed studies. Only one study [101] discussed this, and it was found that folate deficiency post weaning has a protective effect against neoplasia in female $Apc^{Min/+}$ mice, but not in male mice. Whether sex is a strong modifying genetic factor remains unresolved and needs to be investigated with adequately powered studies. To date the majority of the studies have investigated the relative effect of folate deficient and folate adequate diets in the post-weaning phase on small intestinal tumours in the $Apc^{Min/+}$ mouse including such mice with defects in one-carbon or folate–methionine metabolism genes. We collated together the reported numerical data for small intestinal and colon tumour frequency per mouse from these studies (Table 2.2 and Table 2.3) and all together it is evident that there is no significant effect of folate intake on tumour number in the $Apc^{Min/+}$model (Figure 2.4). The limited data on the effect of folate excess also suggest a lack of impact on tumour incidence when data are pooled together (Table 2.2 and Table 2.3). This therefore raises the concern that the results of single small studies may be unreliable on their own and in fact inconsistencies in repeat experiments within the same laboratory using the same $Apc^{Min/+}$ model have been reported [91].

Table 2.2: Mean small intestinal tumour frequency per mouse reported in published studies for $Apc^{Min/+}$ mice fed either folate-deficient,- adequate or -excessive diets during the post-weaning phase (i.e. after the age of 21 days)

Publication	Genotype	Age at diet start	Age at sacrifice	Number of SI tumours per mouse by folate diet		
				Deficient	Adequate	Excessive
Song et al. [94]	$Apc^{Min(+/-)}$	21 days	12 weeks	24	23	19
	$Apc^{Min(+/-)}$	21 days	24 weeks	18	26	21
Song et al. [95]	$Apc^{Min(+/-)}$,$Msh^{(2-/-)}$	21 days	11 weeks	229	ND	110
Sibani et al. [97]	$Apc^{Min(+/-)}$	21 days[a]	13 weeks	16	36	ND
	$Apc^{Min(+/-)}$	21 days[a]	13 weeks	35	24	ND
	$Apc^{Min(+/-)}$	21 days[a]	13 weeks	35	19	ND
Trasler et al. [98]	$Apc^{Min(+/-)}$, $DNMT1^{(+/+)}$	21 days	13 weeks	53	61	ND
	$Apc^{Min(+/-)}$, $DNMT1^{(c/+)}$	21 days	13 weeks	22	20	ND
	$Apc^{Min(+/-)}$, $DNMT1^{(c/+)}$	21 days	13 weeks	26	40	ND
Lawrence et al. [100]	$Apc^{Min(+/-)}$, $Rfc1^{(+/+)}$	21 days	10 weeks	57	60	ND
	$Apc^{Min(+/-)}$, $MTR^{(+/+)}$	21 days	10 weeks	50	33	ND
	$Apc^{Min(+/-)}$, $Rfc1^{(+/-)}$	21 days	10 weeks	42	30	ND
	$Apc^{Min(+/-)}$, $MTR^{(+/-)}$	21 days	10 weeks	43	30	ND
McKay et al. [101]	$Apc^{Min(+/-)}$ [b]	21 days	13 weeks	8	12	ND
	$Apc^{Min(+/-)}$ [c]	21 days	13 weeks	9	14	ND

Publication	Genotype	Age at diet start	Age at sacrifice	Number of SI tumours per mouse by folate diet		
				Deficient	Adequate	Excessive
Lawrence et al. [102]	$Apc^{Min(+/-)}$, $MTHFR^{(+/+)}$	21 days	10 weeks	22	29	41
	$ApcMin^{(+/-)}$, $MTHFR^{(+/-)}$	21 days	10 weeks	25	25	38
MacFarlene et al. [103]	$Apc^{Min(+/-)}$	21 days [a]	14 weeks	24	29	ND
	$Apc^{Min(+/-)}$, $Mthfd1^{(gt/+)}$	21 days [a]	14 weeks	21	21	ND
MacFarlene et. al. [104]	$Apc^{Min(+/-)}$, $Shmt^{(+/+)}$	21 days [a]	14 weeks	39	40	ND
	$Apc^{Min(+/-)}$, $Shmt^{(-/+)}$	21 days [a]	14 weeks	59	31	ND
	$Apc^{Min(+/-)}$, $Shmt^{(-/-)}$	21 days [a]	14 weeks	33	48	ND

Notes: Deficient = 0 mg/kg or 0.2–0.4 mg/kg with sulphathiazole; adequate = 2 mg/kg; excessive = 8 mg/kg or 20 mg/kg. ND = not done.

[a] Folate deficient diets were also deficient in choline.

[b] Maternal diet was folate deficient.

[c] Maternal diet was folate adequate.

Table 2.3: Mean colon tumour frequency per mouse reported in published studies for $Apc^{Min/+}$ mice fed either folate-deficient,-adequate or -excessive diets during the post-weaning phase (i.e. after the age of 21 days)

Publication	Genotype	Age at diet start	Age at sacrifice	Number of SI tumours per mouse by folate diet		
				Deficient	Adequate	Excessive
Song et al. [94]	$Apc^{Min(+/-)}$	21 days	12 weeks	4.6	4.2	3.7
	$Apc^{Min(+/-)}$	21 days	24 weeks	2.6	2.8	3.2
Song et al. [95]	$Apc^{Min(+/-)}$,$Msh^{(2-/-)}$	21 days	11 weeks	1.7	ND	0.6
McKay et al. [101]	$Apc^{Min(+/-)}$ [b]	21 days	13 weeks	0.7	0.3	ND
	$Apc^{Min(+/-)}$ [c]	21 days	13 weeks	0.4	0.3	ND
MacFarlene et al. [103]	$Apc^{Min(+/-)}$	21 days [a]	14 weeks	0.6	1.6	ND
	$Apc^{Min(+/-)}$, $Mthfd1^{(gt/+)}$	21 days [a]	14 weeks	0.7	0.7	
MacFarlene et al. [104]	$Apc^{Min(+/-)}$, $Shmt^{(+/+)}$	21 days [a]	14 weeks	0.6	1.3	ND
	$Apc^{Min(+/-)}$, $Shmt^{(-/+)}$	21 days [a]	14 weeks	2	1	ND
	$Apc^{Min(+/-)}$, $Shmt^{(-/-)}$	21 days [a]	14 weeks	1.2	1.1	ND

Notes: Deficient = 0 mg/kg or 0.2–0.4 mg/kg with sulphathiazole; adequate = 2 mg/kg; excessive = 8 mg/kg or 20 mg/kg. ND = not done.

[a] Folate deficient diets were also deficient in choline.

[b] Maternal diet was folate deficient.

[c] Maternal diet was folate adequate.

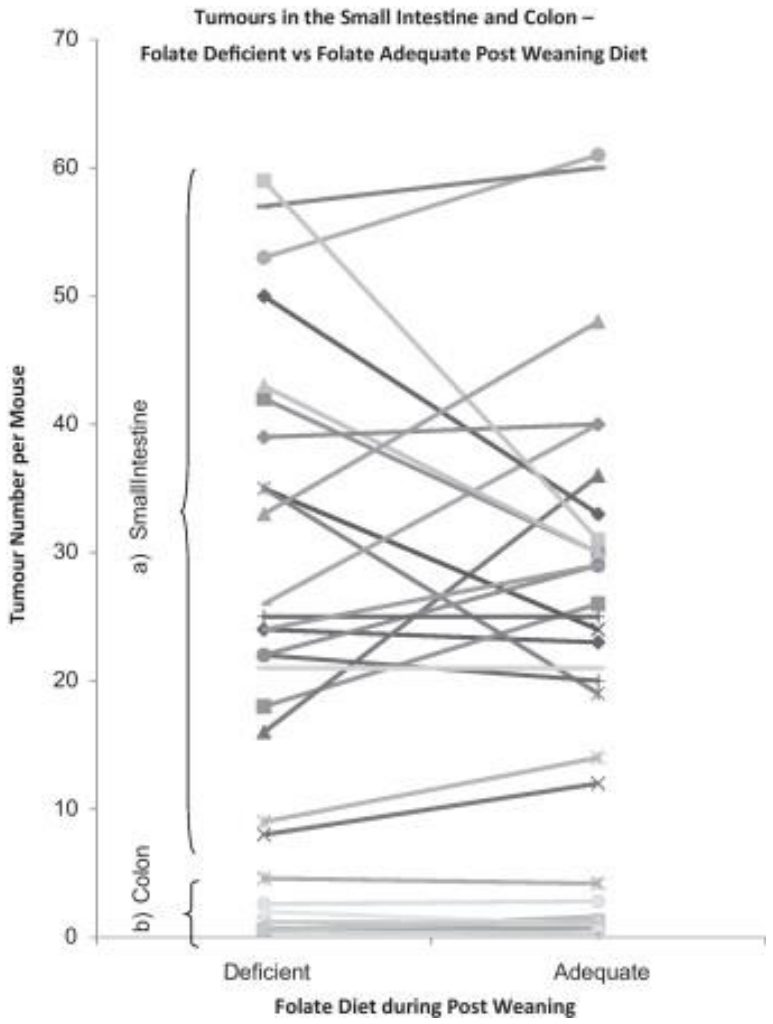


Figure 2.4: (a) Small intestinal tumour frequency per mouse in $Apc^{Min/+}$ mice that were fed folate deficient (0–0.4 mg/kg) or folate adequate diets (2 mg/kg) post-weaning in experiments reported in eight published studies [94, 95, 97, 100-104]. Some studies reported more than one experiment. For further details refer to Table 2.2. (b) Colon tumour frequency per mouse in $Apc^{Min/+}$ mice that were fed folate deficient (0–0.4 mg/kg) or folate adequate diets (2 mg/kg) post-weaning in experiments reported in four published studies [94, 95, 101-104]. Some studies reported more than one experiment. For further details refer to Table 2.3.

Whether the results of experiments in $APC^{Min/+}$ mice on folate and methionine may reflect the effects on intestinal cancer in wild type or genetically normal mice is unclear. However, a study by Newmark et al. [136] showed increased intestinal neoplasms in normal C57/BL6 mice that were fed a "western-style diet" that was reduced in calcium, vitamin D, folic acid, methionine, choline and vitamin B12 to an extent approximating that consumed by western human populations. Subsequent studies by the same group using this model showed that the "western-style diet" induced changes in gene expression in colonic epithelium similar to that caused by inheritance of a mutant APC gene [137]. Furthermore, these studies showed that supplementation of the "western style diet" with calcium and vitamin D suppressed colon tumours but there was no benefit following supplementation with folic acid, methionine, choline and vitamin B12 [138].

Variability in the reviewed studies including number of mice used, model system and different diet contributes to the conflicting outcomes. There were quite a lot of variability between studies and even in one reviewed study when the study was repeated, which suggest the need for a stricter and statistically more robust study design to improve the probability that results can be reproduced consistently within and across laboratories. Statistical power analysis is important to estimate the minimum sample size required to reasonably detect an effect [139]. Based on the original work by Moser et al. [85], total average tumour number per mouse is 29 ± 10 (mean ± standard deviation). A sample size of 14 mice per group gives a statistical power of 82% for a one tailed test at the 5%

significance level to detect a 30% decrease in the number of tumours. Statistical analysis used in the reviewed studies were mostly analysis of variance (ANOVA) and correlation test.

The conflicting data from individual small studies on the effect of folate on tumorigenesis requires further investigation with statistically more robust designs, including measurements of folate derivatives and other relevant indicators (such as expression of folate and methionine receptors). The scarcity of data on the (possibly harmful) effects of excess dietary methionine in APC defective genetic backgrounds emphasises the need for further investigation and whether this is influenced by the methionine-dependence phenotype resulting from deletion of methylthioadenosine phosphorylase (MTAP) gene required for regeneration of methionine in cells [140]. Furthermore, more attention should be given to other mechanisms by which folate and methionine affect gene expression; for example folate deficiency or excess can alter histone methylation and microRNA profiles but the extent to which these factors play a role in intestinal carcinogenesis in $APC^{Min/+}$ mice remains unclear [141, 142]. Therefore, more folate and methionine-dependent tumorigenesis mechanisms should be studied, such as changes in histone and DNA methylation status, assessment of chromosomal stability and DNA damage, the influence of SAM: SAH ratio, interaction between APC and polyamines, and DNA methyltransferase expression and activity. These may provide firmer evidence as to whether folate and/or methionine will be tumour promoting or tumour protective in this model. The impact of folate and/or methionine on tumour cell proliferation will ultimately

depend on the unique genetics that evolves within each intestinal tumour, which may explain some of the inconsistencies observed between and within experiments.

2.8 Recent Updates

There are two recent studies with similar interest that have been reported since the above review was published in 2012 [93]. Studies by Kadaveru et al. [143] and Hanley et al. [144] showed promising effect of methyl donor deficiency (folate deficiency, in combination with choline, methionine and vitamin B12 depletion) in reducing small intestinal tumour (by ~96%) in $Apc^{Min}/+$ model [143] and total number of polyps (by 78%) in $Apc^{\Delta 14}/+$ mice model [144]. Interestingly, temporary dietary methyl donor deficiency imparts continuing tumour protection to the intestine. This was evidenced by reduced intestinal tumour (by 68%) in mice groups that were initially given methyl donor deficient diet for a few weeks and then given methyl donor sufficient diet until end of study in Hanley et al. [144].

In further understanding of mechanisms involving folate, methionine and Apc background in colorectal cancer there are a few interesting insights that have been reported more recently. In a study on ornithine decarboxylase inhibitor α-difl uoromethylornithine (DFMO, which is an effective chemopreventive agent for colorectal cancer) actions on colon cancer cell metabolism, it was observed that DMFO treatment on Apc^{Min} intestinal tumors and human colorectal cancer cells HT29 is associated with reduced levels of folate-dependent metabolites, including SAM, thymidine pools, and related

pathway intermediates including decreased levels of polyamine pathway products, spermine and spermidine [145].

Obesity increases intestinal cancer risk [146-148] through activation of WNT signalling [146, 147]. WNT signalling pathway is observed to be activated in $Apc^{+/1638N}$ mice, that were induced to become obese either by diet or genetically [147]. The activation was also observed in diet-induced obesity in C57BL/6 mice, through increased expression of TNF-α in colon [146]. These observations indicate that WNT signalling activation is responsible in increasing intestinal cancer in obesity [146, 147].

One of the limitation of the $Apc^{Min/+}$ model is that tumour mainly develops in the small intestine whereas in human intestinal cancer develops mainly in the colon [94]. To improve the model and increase the similarity to human condition, Cre-Lox system has been employed [149]. Colonic tumour increased by expressing Cre in the intestinal tract [149-152]. This offers a better $Apc^{Min/+}$ model in future studies for understanding the effects of dietary factors in colorectal cancer in human.

Interest on transgenerational effect of methyl donor in the diet has expanded recently. Apart from the two studies by Mckay [101, 105] on maternal intake discussed in the above review [93], there is a recent study by Sabet et al. [153] who explored the paternal intake of male Apc^{1638N} mice. They found that modulation of paternal vitamins B2, B6, B12, and folate intake alters tumour volume in the offspring in gender-specific manner.

The female offspring developed higher tumour volume if the father had higher intake of vitamins B2, B6, B12, and folate [153].

Chapter 3:

Hypotheses and Objectives

3.1 Hypotheses

Overarching hypothesis:

Genome stability and cell growth of intestinal cells, including cancer cells, is modified by deficiency or excess of methyl donor micronutrients such as folate and methionine.

Specific hypotheses tested for *in vitro* study:

1. Restriction of folic acid or methionine prevents excessive proliferation of HT29 cells.
2. Excess of folic acid or methionine promotes DNA stability in HT29 cells.
3. Folic acid or methionine deficiency are associated with increased telomere length in HT29 cells.
4. Folic acid or methionine deficiency result in decreased DNA methylation in HT29 cells.

Specific hypotheses tested for *in vivo* study:

1. Excess dietary folic acid and/or methionine decreases cancer incidence and growth in the small intestine and colon of the $Apc^{Min/+}$ model.
2. Dietary folic acid and methionine promote DNA stability in the $Apc^{Min/+}$ mouse model.

3.2 Aims

Aims of this thesis were to provide satisfactory answers to the knowledge gaps on the role of folate and methionine in colon cancer initiation and progression by assessing DNA stability and tumour incidence. Studies were performed *in vitro* (using human colorectal adenocarcinoma HT29 cell line) and *in vivo* (using $Apc^{Min/+}$ mouse model). This research may have relevance in developing nutritional guidelines for folate and methionine applicable for humans.

Specific objectives of this thesis were:

1. To investigate the effects of folic acid and/or methionine concentration on growth and genomic stability in the HT29 cell line (*in vitro*).
2. To investigate the effects of folic acid and/or methionine dietary intake on DNA damage and colon cancer incidence in the cancer prone $Apc^{Min/+}$ mouse model (*in vivo*).

Chapter 4:

In Vitro Studies – I

The Effect of Folic Acid and Methionine on Cell Growth and Genome Stability in HT29 Cells

4.1 Objective

This aspect of study was carried out to investigate the effects of various folic acid and methionine concentrations on the cell proliferation and genomic instability of HT29 cell lines.

4.2 Hypotheses

1. Restriction of folic acid or methionine prevents excessive proliferation of HT29 cells.
2. Excess of folic acid or methionine promotes DNA stability in HT29 cells.

4.3 Introduction

Cell culture based screening is considered to be the first step in screening the potential of any nutrients for cancer prevention and control. *In vitro* cell culture models have been recognized to be valuable in the investigation of cell proliferation [71] and DNA stability [154]. Thus, this part of study was carried out using an *in vitro* human colorectal adenocarcinoma model, i.e. the HT29 cell line. HT29 cells are the most widely used *in vitro* model for human colon epithelial cells [155]. It is derived from the colon of a 44 years old Caucasian female with colon adecarcinoma (ATCC HTB-38, Manassas, VA, USA).

Evidence is lacking from previous studies looking directly at the effect of folate and methionine in HT29. A study by Pellis et al. (2008) [156] investigated the process affected by different folate concentration in HT29; supplementation of HT29 cells with 100 ng/mL folic acid (in the presence of vitamin B12 and other micronutrients involved in the folate–methionine cycle) resulted in higher intracellular folate levels, cell growth, apoptosis and metabolic activity compared to supplementation with 10 ng/mL folic acid. At 100 ng/mL folic acid, higher proliferation and apoptosis were observed, whereas gene expression analysis and lower E-cadherin protein expression indicated decreased differentiation. These are supporting the promoting effect of folic acid supplementation on established colorectal neoplasms [156]. A study by Kulcsár et al. (2013) [157] demonstrated that methionine as part of amino acids mixture (consists of L-arginine, L-histidine, L-methionine, L-phenylalanine, L-tyrosine, L-tryptophan, L-ascorbate, D-biotin, pyridoxine, riboflavin, adenine, L(-)malate) inhibits the growth of HT29. Luo et al. (2010) [158] showed that HT29 cell growth could be inhibited with addition of SAM. SAM was shown to inhibit tumor cell growth by reversing the DNA hypomethylation of promoters of oncogenes, therefore down-regulating their expression [158].

From the perspective of DNA stability, previous evidence showed that folic acid deficiency induces genomic instability measured as micronuclei, nucleoplasmic bridges and nuclear buds *in vitro* (in human lymphocytes), which may contribute to the initiation and/or development of cancers [159]. Methionine deficiency or excess, in contrast, has not been

shown to impact on genomic stability in human lymphocytes when treated with 0 to 0.1 mM L-methionine [160]. Rodent studies, however, have shown that methionine has impact on genomic stability ([126, 161] and as reviewed in [162]). Rats fed with methyl-deficient diet (deficient in the methyl donors methionine, choline, and folic acid) for 9 weeks showed increase DNA strand breaks incidence [126]. Conflicting data from a study by Aissa et al. (2013) [161], showed that changes in methionine intake resulted in a tissue-specific effect on DNA damage in mice fed a healthy diet with respect to all other micronutrients. They found that excess methionine (2% of DL-methionine supplementation) induced chromosomal and DNA damage in peripheral blood, while methionine deficiency (0% DL-methionine supplementation) reduced basal DNA damage in the liver [135].

4.4 Methods and Preliminary Experiments

4.4.1 HT29 Cell Culture

Human epithelial cells isolated from a primary colorectal adecarcinoma, HT29 (ATCC HTB-38, Manassas, VA, USA), were seeded in 75cm^2 culture flasks (Thermo Fisher Scientific, NY, USA), and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, St. Louis, MO, USA) (93%) supplemented with 5% (v/v) foetal bovine serum (FBS) (Thermo Trace, Australia), 1% 1 mM L-glutamine (Sigma, USA) and 1% (v/v) penicillin (5000 IU/mL)/streptomycin (5 mg/mL) (Sigma, USA). Cells were maintained in incubator at 37°C with 5 % CO_2 and 100 % relative humidity. Culture medium was replaced with fresh medium every 2 days. Cells were split or harvested at 90 % confluence.

To remove the adherent HT29 cells, 1 mL of 0.25% trypsin (Sigma, USA) was pipetted into the culture flask and distributed thoroughly. After 5 minutes, 5 mL of phosphate-buffered saline (PBS) were added and cells were then collected through centrifugation at 100Xg for 5 minutes. Cells were then seeded for further use.

4.4.2 Preparation of Medium with Different Folic Acid and Methionine Concentrations

Different concentrations of folic acid and methionine in media were used in this study:

- Control: RPMI 1640 medium (containing 2655 nM/L folic acid, 100.6 µM/L methionine)
- Folic acid: 4, 10, 20, 50 & 100 nM/L folic acid
- Methionine: 4, 10, 20, 50 & 100 µM/L methionine

To achieve the different concentrations needed, four basic media were prepared as follows: (i) RPMI 1640 with 100 nM folic acid concentration, (ii) RPMI 1640 with 0 nM folic acid concentration, (iii) RPMI 1640 with 100 µM methionine concentration, and (iv) RPMI 1640 with 0 µM methionine concentration. RPMI 1640 medium (folate-free, methionine-free and complete) were obtained from Sigma (St. Louis, MO, USA). RPMI 1640 medium (Sigma, St. Louis, MO, USA) (93%) were supplemented with 5% (v/v) foetal bovine serum (FBS) (Thermo Trace, Australia), 1% 1 mM L-glutamine (Sigma, USA) and 1% (v/v) penicillin (5000 IU/mL)/streptomycin (5 mg/mL) (Sigma, USA). Because 5% FBS may contribute a small amount of folate and methionine and to take account of possible increases in addition

of folic acid or methionine, prepared medium was sent to South Australia Pathology, Adelaide, South Australia to measure the actual folate and methionine concentrations. Based on the measured concentration, the desired concentrations of folic acid and methionine were achieved by mixing a high concentration medium diluted with medium lacking the constituent.

For example, to prepare a 100 mL of 4nM folic acid concentration media:

Two basic media were required:

A. RPMI 1640 with 100 nM folic acid concentration
B. RPMI 1640 with 0 nM folic acid concentration

99.602 mL of B was combined with 0.398 mL of A to achieve a final concentration 4nM folic acid in a 100 mL media.

The concentration of 4-20 nM folic acid and 4-20 µM methionine were considered as low or deficient for cell growth and/or genome stability. 50 nM folic acid and 50 µM methionine were considered as closer to the optimal physiological range for human, and 100 nM folic acid and 100 µM methionine were considered as higher than optimal requirement. Previous studies in human lymphocytes have shown that folic acid concentration below 20 nM decreases cells proliferation and increases DNA damage and methionine concentration lower than 20 µM inhibits cell growth and viability [159, 163].

4.4.3 HT29 Cell Proliferation

4.4.3.1 Growth Observation of 14 Day Culture of HT29 in 96 Well Plates

A growth curve (Figure 4.1) of HT29 over 14 days duration was generated, to determine an optimal seeding density to be used for the main study. This was done to ensure that the cell density was not too high and cells did not reach the plateau of the growth curve until day 14.

HT29 cultures were established using control (RPMI) medium, at a total volume of 100µL per well, in a 96-well plate (Thermo Fisher Scientific, NY, USA). Seeding concentrations were set at $1X10^5$/mL, $1X10^4$/mL, $1X10^3$/mL, $5X10^2$/mL, $1X10^2$/mL, and 1X10/mL. Cultures were established in triplicate (3 wells for each concentration) and 8 identical plates were set up for MTT assay. Cells were maintained in control (RPMI) medium, incubated at 37°C with 5 % CO_2 and 100 % relative humidity. Culture medium was replaced every alternate day, by removing 30µL of medium from each well and replaced with fresh medium.

Cells proliferation was determined by MTT assay [164] for day 0, 1, 3, 5, 8, 10, 12, and 14 of incubation. Based on Figure 4.1, cells seeding concentration of $5X10^2$/mL was selected for the main experiments.

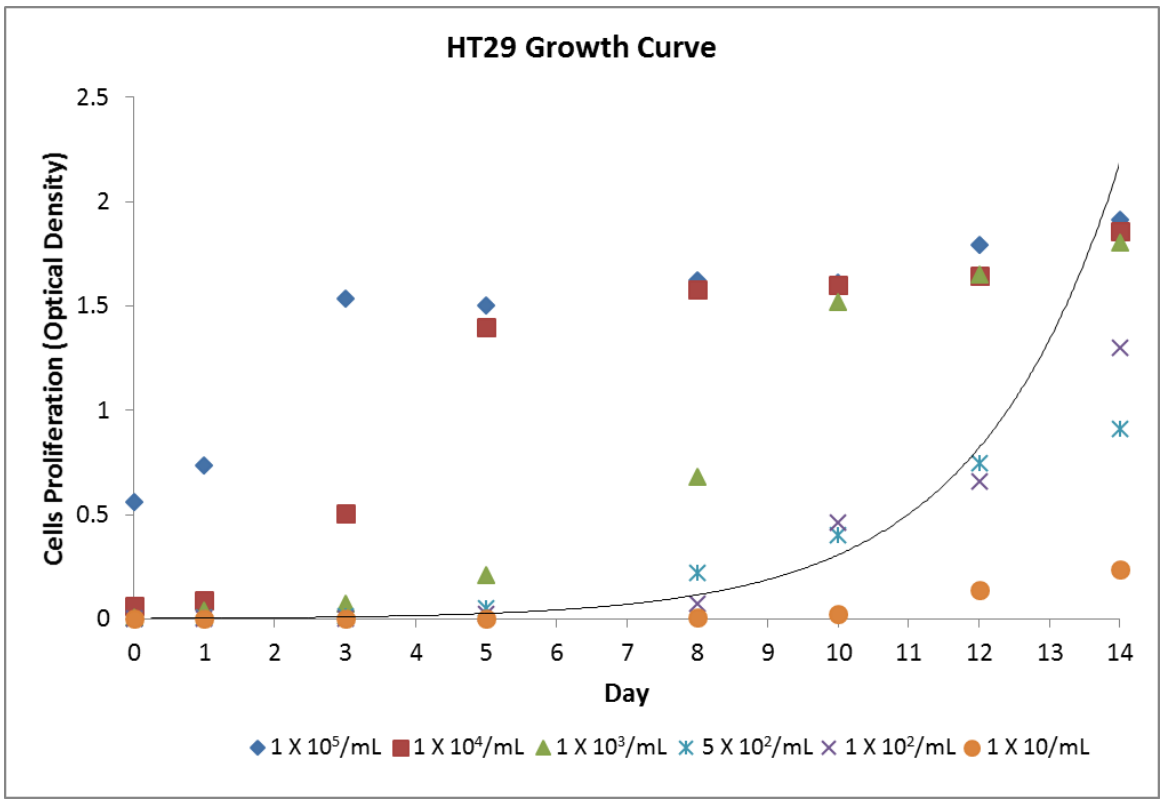


Figure 4.1: HT29 growth curve over 14 days. Data expressed as mean of triplicate wells. The symbols represent the initial concentration of cells seeding per mL. A growth curve plotted to determine the growth pattern. Based on the growth curve, cells seeding concentration of $5X10^2$/mL was selected for the main experiments, to ensure that the cell density was not too high and cells did not reach the plateau of the growth curve until day 14.

4.4.3.2 HT29 Cell Proliferation in Various Folic Acid and Methionine Concentrations

100μL of HT29 cells were seeded at a concentration of $5X10^2$/mL per well in a 96-well plate (Thermo Fisher Scientific, NY, USA). Cells were maintained in different concentrations of folic acid and methionine (prepared as detailed in 4.4.2), for 14 days.

All medium (Sigma, St. Louis, MO, USA) (93%) were supplemented with 5% (v/v) foetal bovine serum (FBS) (Thermo Trace, Australia), 1% 1 mM L-glutamine (Sigma, USA) and 1% (v/v) penicillin (5000 IU/mLl)/streptomycin (5 mg/mL) (Sigma, USA). Cells were maintained in incubator at 37°C with 5 % CO_2 and 100 % relative humidity. Culture medium was replaced every alternate day, by removing 30μL of medium from each well and replaced with fresh medium. The experiment was carried out in six replications (6 wells for each treatment) using a culture period of 14 days duration and 12 identical plates were set up. Cells proliferation was measured with i) MTT assay on day 0, 3, 7, 9, 11 and 13 [164] and ii) Crystal Violet dye elution on day 1, 3, 7, 9, 11 and 13 [165].

The MTT assay is based on the basic principle of mitochondrial activity in most viable cells is constant. Thus, an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The conversion of the tetrazolium salt MTT into formazan crystals which can be solubilised for homogenous measurement, reflects the mitochondrial activity of the cells. Thereby, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) [166]. Meanwhile in

Crystal Violet dye elution assay, the cell number is reflected by the nuclear DNA content measured photometrically after solubilization of Crystal Violet dye that accumulates in the cell nucleus [167].

From MTT and Crystal Violet dye elution results, growth rate percentage was calculated based on formulation as below:

$$\left[\frac{\text{(OD reading of day n – OD reading 48 hours earlier)}}{\text{OD reading 48 hours earlier}} \right] \times 100$$

Example:

Growth rate % of day 9=

[(OD of day 9 – OD of day 7)/ OD of day 7] X 100

i) MTT Assay [164]

10 µl of 5mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) salt was added to each well and incubated for 2 hours at 37° C. When purple precipitate was clearly visible under the microscope, 100 µL of solubilizing solution was added (10% Sodium Dodecyl Sulphate (Sigma, St. Louis, MO, USA) in 0.01 M HCl (BDH, Analar, England)). The plate was then placed in the incubater overnight. OD/absorbance was measured with ELISA microplate reader (SpectraMax 250, Molecular Devices, CA, USA) at 650nm and 570nm. Average OD values were determined for each

condition and the average value for the blank wells subtracted. A higher OD reading indicated higher proliferation.

ii) Crystal Violet dye elution [165]

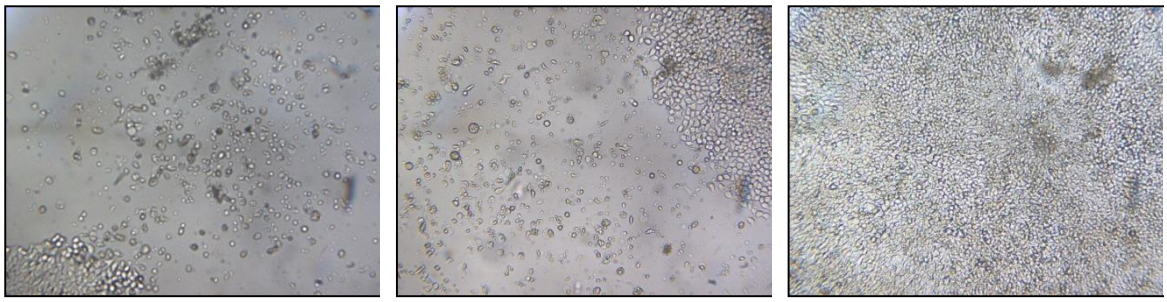
All media was removed, and the plate was rinsed with 100μL of PBS in each well. Each well was stained with 50μL 0.5% Crystal Violet solution (Sigma, St. Louis, MO, USA). After 10 minutes, stain was removed by rinsing with water four times. The plate was then placed in the incubator to allow drying. 100μL acetic acid solution (33%) (BDH, Analar, England) was added to each well to de-stain. OD was measured with ELISA microplate reader (SpectraMax 250, Molecular Devices, CA, USA) at 570nm. OD values were determined for each condition and the average value for the blank wells subtracted. A higher OD reading indicated higher proliferation.

4.4.4 Genomic Stability and Cytostasis of HT29 Cells

4.4.4.1 Preliminary Analysis of Growth Observation of 14 Day Cultures of HT29 in 24 Well Plates

DNA stability measurement of HT29 cell culture was carried out on cells cultured in 24 well plates over 14 days. Preliminary studies of cell growth were conducted to determine an appropriate seeding concentration to be used in investigating DNA stability. This was done to ensure that the cells culture was not overly populated on day 14, to allow a sufficient number of dividing cells to be present on day 14 to perform the Cytokinesis-block micronucleus cytome (CBMN Cyt) assay.

HT 29 cells were seeded in 24-well plate (Thermo Fisher Scientific, NY, USA) at 1mL volume of control (RPMI) medium per well. Seeding concentrations were set at $1X10^3$/mL, $1X10^2$/mL, and 1X10/mL. Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) (93%) supplemented with 5% (v/v) foetal bovine serum (FBS) (Thermo Trace, Australia), 1% 1 mM L-glutamine (Sigma, USA) and 1% (v/v) penicillin (5000 IU/mL)/streptomycin (5 mg/mL) (Sigma, USA). Cells were placed in incubator at 37°C with 5 % CO_2 and 100 % relative humidity. Culture medium was replaced every alternate day, by removing 500μL of medium from each well and replaced with fresh medium. Cells were observed visually under microscope throughout the 14 days duration. Based on observation on the cells confluency (Figure 4.2), seeding concentration at $1X10^2$ cells/mL was chosen.



A B C

Figure 4.2: Growth observation of 14 day cultures of HT29 in 24 well plates (10 X objective). A: seeding concentration of 1X10/mL, B: seeding concentration of $1X10^2$/mL, and C: seeding concentration of $1X10^3$/mL. Based on observation on confluency, seeding concentration at $1X10^2$ cells/mL was used for the main experiment, to allow a sufficient number of dividing cells to be present on day 14 to perform the CBMN Cyt assay.

4.4.4.2 DNA Stability in HT29 in Various Folic Acid and Methionine Concentrations

HT29 cells were seeded at $1X10^2$/mL per well in a 1 mL volume in 24-well plate. Cells were cultured in different concentrations of folate and methionine, using culture medium prepared as detailed in 4.4.2, for 14 days.

Cells were maintained in an incubator at 37°C with 5 % CO_2 and 100 % relative humidity. Culture medium was replaced every alternate day, by removing half volume of medium from each well (500μL) and replaced with fresh medium. Genome stability was determined by scoring biomarkers of DNA damage using the CBMN Cyt assay (as detailed in 4.4.5) [168]. Experiments were done using three replicates of cultures for each treatment and repeated six times.

4.4.5 CBMN Cyt Assay [168]

i) Methods

Cells were harvested on day 14 of the culture for each experiment. 24 hours prior to harvesting, Cytochalasin-B (Cyto-B, Sigma, St. Louis, MO, USA) was added to stop dividing cells from undergoing cytokinesis. 74.67μL of medium was removed from each well and replaced with the same volume of Cyto-B, to give a final concentration of 4.5μg/mL of Cyt-B.

24 hours after addition of Cyto-B, all medium was removed from each well. 200μL TripLE™ Express (Invitrogen, CA, USA) was added to each well and left for 10 minutes until

the adherent cells were released. 200μL of fresh culture medium was then added to each well. Resuspended cells were harvested onto microscope slides by pipetting 150μL of cell suspension from each well into a cytocentrifuge sampling cup, and spun at 600 rpm for 5 minutes in a cytocentrifuge (Shandon Cytospin 3, Shandon Products, UK). The slides were then turned and another 150μL of cell suspension were added into the sampling cup, and spun to get two spots on each slide. Slides were air-dried for 10 minutes prior to fixation in Diff-Quik fixative for 10 minutes, and stained with Diff-Quik (Lab Aids, Australia).

ii) Scoring

Slides were examined using a transmitted light microscope (Leica, Wetzlar, Germany) under 1000X magnification (10 X eyepiece magnification, 100 X oil immersion objective). A total of 500 cells were scored to determine whether they were mononucleated, binucleated (BN), and multinucleated to derive the nuclear division index (NDI), as well as observing the frequency of cells undergoing apoptosis and necrosis. A total of 1000 BN cells were scored to determine the frequency of DNA damage biomarkers (micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs)) [168].

iii) Scoring Criteria

The scoring criteria were as defined by Fenech, 2007 for primary lymphocytes [168]:

a) Criteria for scoring mononucleated, binucleated (BN), and multinucleated cells [168]

Frequency of viable mononucleated, binucleated and multinucleated cells was measured to determine cytostatic effects and the rate of mitotic division, calculated using the NDI. The NDI was calculated as follows:

$$NDI = \frac{[(\text{number of mononucleated cells scored X 1}) + (\text{number of BN cells scored x 2}) + (\text{number of of multinucleated cells scored X 4})]}{\text{total number of cells examined}}$$

These cell types have the following characteristics:

- Mono-, bi and multinucleated cells are viable cells with an intact cytoplasm and normal nucleus morphology containing one, two and three or more nuclei, respectively.
- They may or may not contain one or more MNi or NBUDs and in the case of bi- and multinucleated cells they may or may not contain one or more NPBs. Necrotic and apoptotic cells were not included among the viable cells scored.

b) Criteria for scoring apoptotic cells [168]

Apoptotic cells are cells undergoing programmed cell death. They have the following characteristics:

- Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear membranes.
- Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.
- Staining intensity of the nucleus, nuclear fragments and cytoplasm in both kinds of apoptotic cell is usually greater than that of viable cells.

c) Criteria for scoring necrotic cells [168]

Necrosis is an alternative form of cell death that is thought to be caused by damage to cellular membranes, organelles and/or critical metabolic pathways required for cell survival such as energy metabolism. Necrotic cells have the following characteristics:

- Early necrotic cells can be identified by their pale cytoplasm, the presence of numerous vacuoles (mainly in the cytoplasm and sometimes in the nucleus), damaged cytoplasmic membrane and a fairly intact nucleus.
- Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary.
- Staining intensity of the nucleus and cytoplasm in both types of necrotic cell is usually less than that observed in viable cells.

d) Criteria for selecting BN cells suitable for scoring MNi, NPBs and NBUDs [168]

The cytokinesis-blocked BN cells that may be scored for MNi, NPB and NBUD frequency should have the following characteristics:

- The cells should be binucleated.
- The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
- The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
- The two nuclei within a BN cell may be attached by a nucleoplasmic bridge, which is no wider than 1/4th of the nuclear diameter.
- The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
- The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

e) Criteria for scoring MNi [168]

MNi are morphologically identical to but smaller than nuclei. They also have the following characteristics:

- The diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.
- MNi are non-refractile and they can therefore be readily distinguished from artifact such as staining particles.
- MNi are not linked or connected to the main nuclei.
- MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

f) Criteria for scoring NPBs [168]

An NPB is a continuous DNA-containing structure linking the nuclei in a binucleated cell. NPBs originate from dicentric chromosomes (resulting from misrepaired DNA breaks or telomere end fusions) in which the centromeres are pulled to opposite poles during anaphase. They have the following characteristics:

- The width of an NPB may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell.
- NPBs should also have the same staining characteristics as the main nuclei.

- On rare occasions, more than one NPB may be observed within one binucleated cell.
- A binucleated cell with an NPB may contain one or more MNi.
- BN cells with one or more NPBs and no MNi may also be observed.

g) Criteria for scoring NBUDs [168]

An NBUD represents the mechanism by which a nucleus eliminates amplified DNA and DNA repair complexes. NBUDs have the following characteristics:

- NBUDs are similar to MNi in appearance with the exception that they are connected with the nucleus via a bridge that can be slightly narrower than the diameter of the bud or by a much thinner bridge depending on the stage of the extrusion process.
- NBUDs usually have the same staining intensity as MNi.
- Occasionally, NBUDs may appear to be located within a vacuole adjacent to the nucleus.

If it is difficult to determine whether the observed nuclear anomaly is an MN touching the nucleus or a nuclear bud, it is acceptable to classify it as the latter.

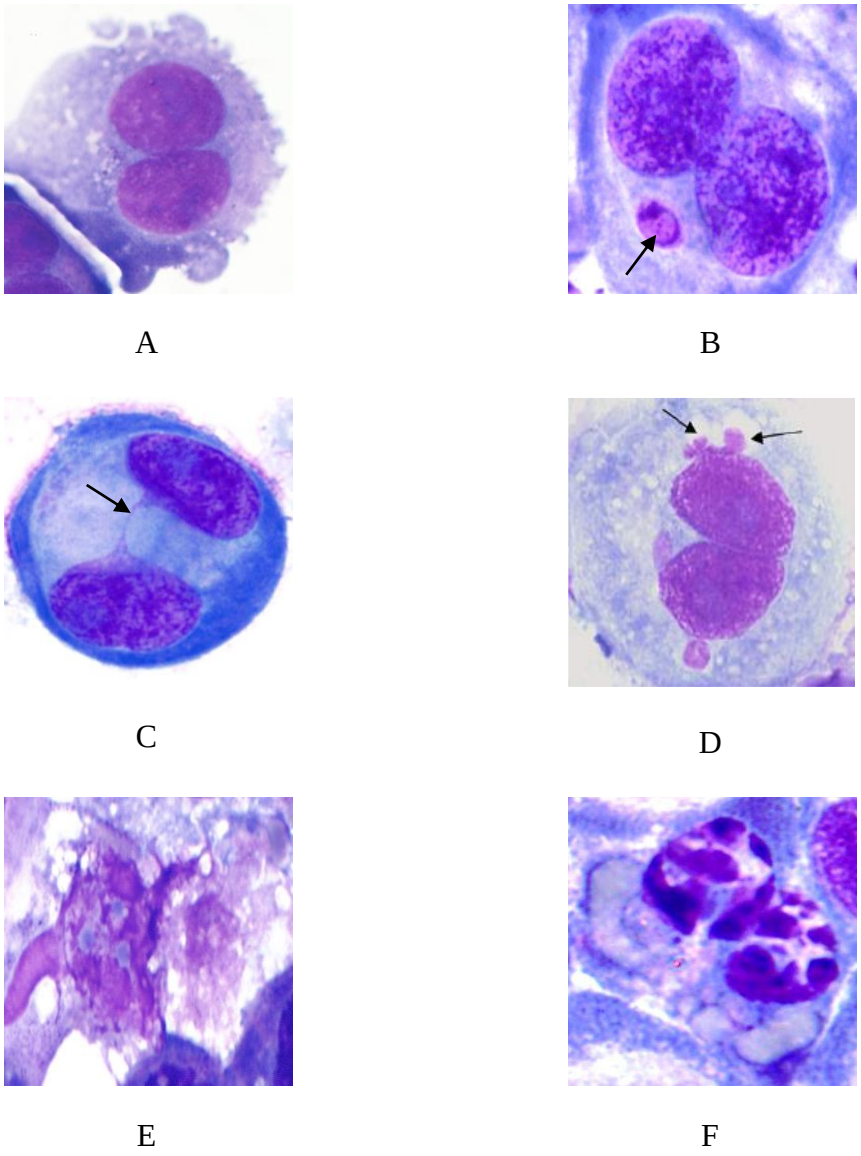


Figure 4.3: Photographic examples of HT29 cells scored with CBMN Cyt assay.

A: Normal binucleated cells, B: Binucleated cells with MNi, C: Binucleated cells with NPBs,

D: Binucleated cells with NBUDs [169], E: Necrotic cells, F: Apoptotic cells.

4.4.6 Statistical Analyses

All data were analyzed with IBM SPSS Statistic 21. Treatment effects were analyzed by repeated measure with Post Hoc Multiple Comparison Bonferroni Test to determine significance of difference between treatments. Analysis of variance (ANOVA) was performed to normal distributed data and for the data that was not normally distributed, ANOVA was performed to log transformed data. Post test for linear trend was performed in GraphPad Prism 7. Significance was accepted at $p < 0.05$.

4.5 Results

4.5.1 HT29 Cell Proliferation

The growth and proliferation of HT29 cells was measured via MTT assay and Crystal Violet dye elution. The MTT assay is based on the conversion of MTT into formazan crystals by living cells. This determines mitochondrial activity, which is related to the number of viable cells [166]. Meanwhile Crystal Violet visualizes DNA, which is related to the total number of cells but does not distinguish between viable and non-viable cells [170].

Figure 4.4 and 4.5 demonstrate HT29 cell proliferation when exposed to various concentrations of folic acid (at 4, 10, 20, 50 and 100 nM/L) and methionine (at 10, 20, 50 and 100 µM/L) over 14 days, measured with MTT assay. Figure 4.6 and 4.7 show cell proliferation when exposed to various concentrations of folic acid and methionine over 14 days, measured with Crystal Violet dye elution assay. Control culture medium (RPMI1640), containing supraphysiological concentration of folic acid (2655 nM) and methionine (100.6 µM) (as per product information provided by manufacturer - Sigma, St. Louis, MO, USA) was used as comparison.

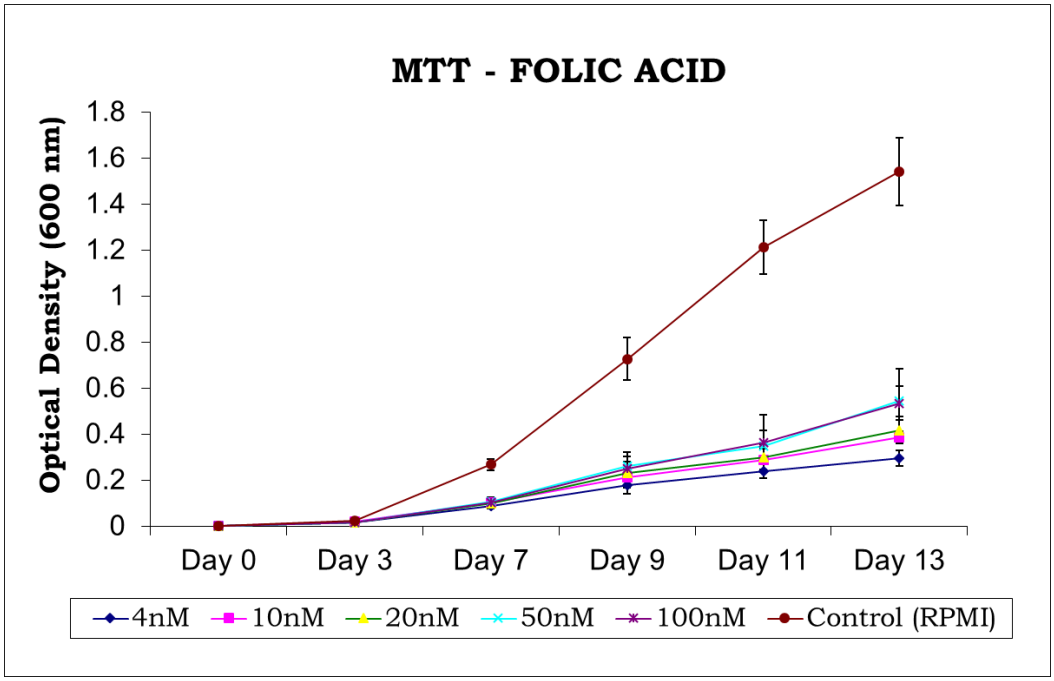


Figure 4.4: HT29 cell proliferation over 14 days with different folic acid concentrations, measured with MTT assay. Data expressed as mean of six replication cultures ± standard error (SE). OD value of 0.001735 is equivalent to 500 cells count with approximately 90% viability (i.e. 450 viable cells).

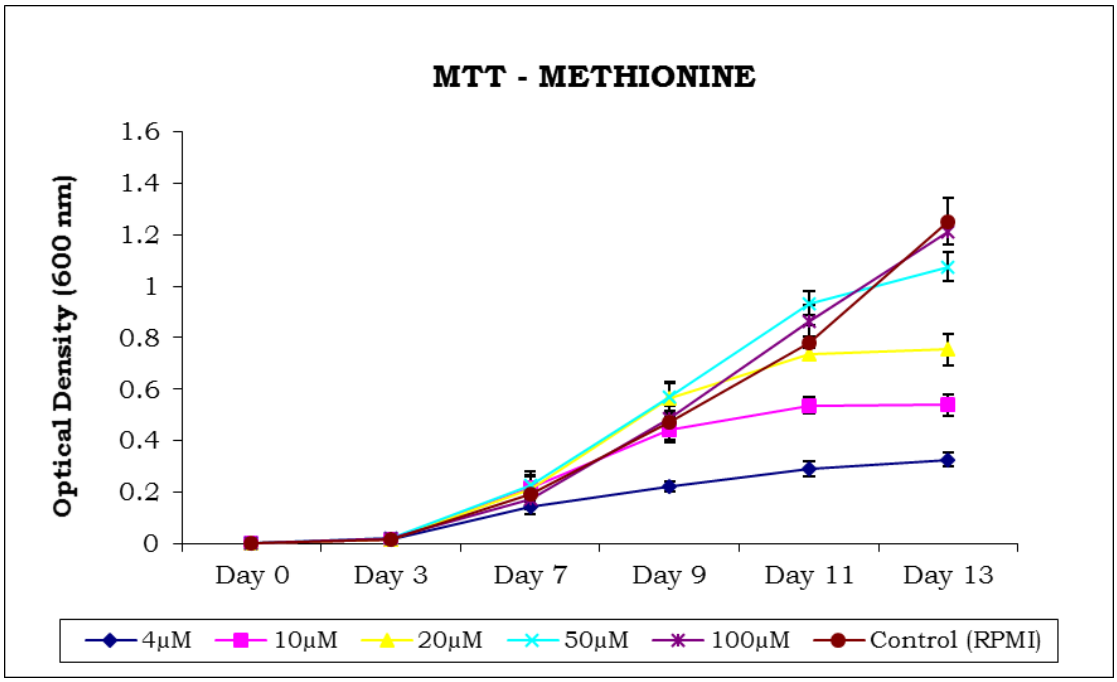


Figure 4.5: HT29 cell proliferation over 14 days with different methionine concentrations, measured with MTT assay. Data expressed as mean of six replication cultures ± SE. OD value of 0.001735 is equivalent to 500 cells count with approximately 90% viability (i.e. 450 viable cells).

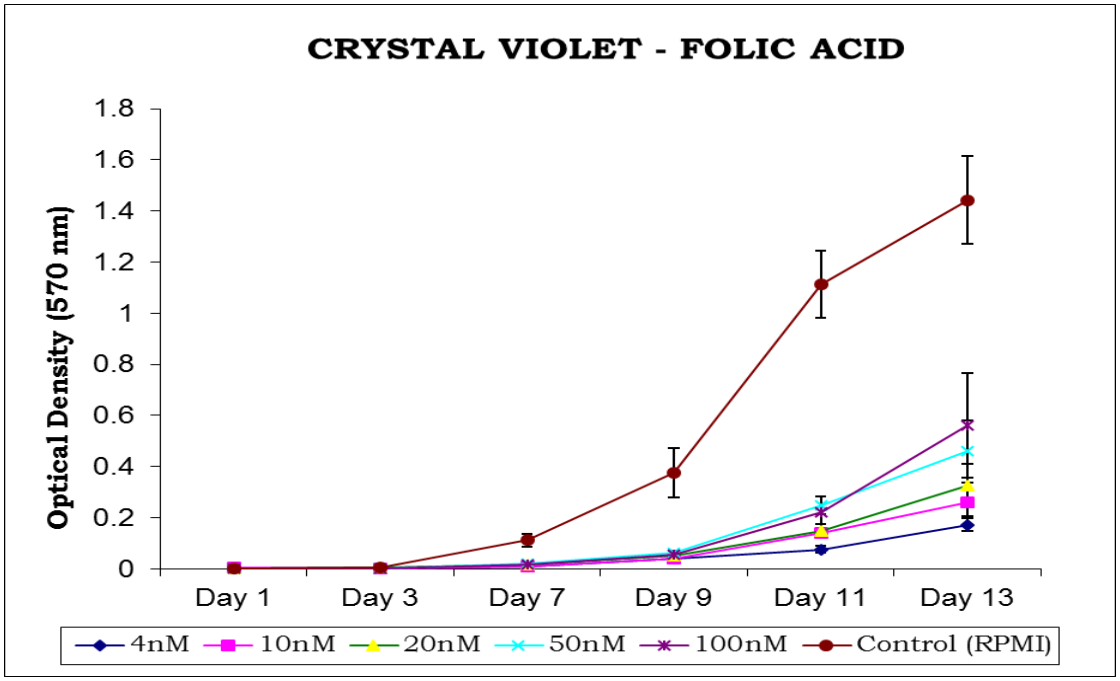


Figure 4.6: HT29 cell proliferation over 14 days with different folic acid concentrations, measured with Crystal Violet dye elution. Data expressed as mean of six replication cultures ± SE. OD value of 0.001719 is equivalent to 500 cells count with approximately 90% viability (i.e. 450 viable cells).

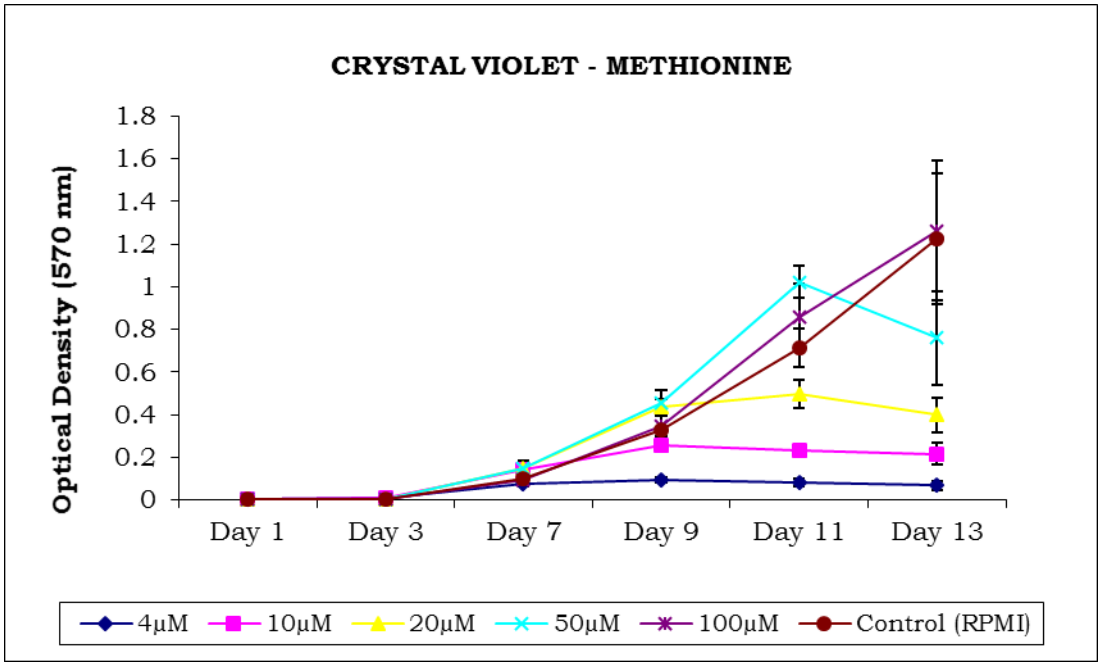


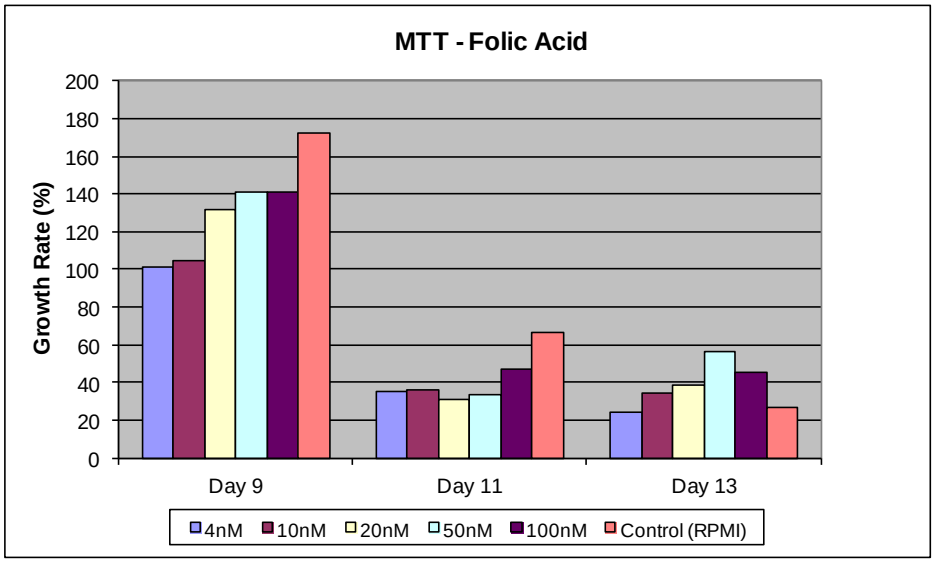
Figure 4.7: HT29 cells proliferation over 14 days with different methionine concentrations, measured with Crystal Violet dye elution. Data expressed as mean of six replication cultures ± SE. OD value of 0.001719 is equivalent to 500 cells count with approximately 90% viability (i.e. 450 viable cells).

Our results are in agreement with previous data of Pellis et al. (2008) showing that HT29 cells are rapidly growing tumour cells in normal (RPMI) medium [156]. When adapting the HT29 cells from normal culture media to the different treatments, a difference in growth was observed. The cell growth reduced with decreasing concentrations of folic acid and methionine, in a dose-dependent manner. Figure 4.4 and 4.6 showed that HT29 exposed to different folic acid concentration continue to proliferate at day 13 (OD range 0.17 – 0.56), but at a substantially lower rate than that of the cells maintained in control RPMI containing 2655 nM folic acid (mean OD of 1.54 for MTT and 1.44 for Crystal Violet dye elution), with p value = 0.00 – 0.01.

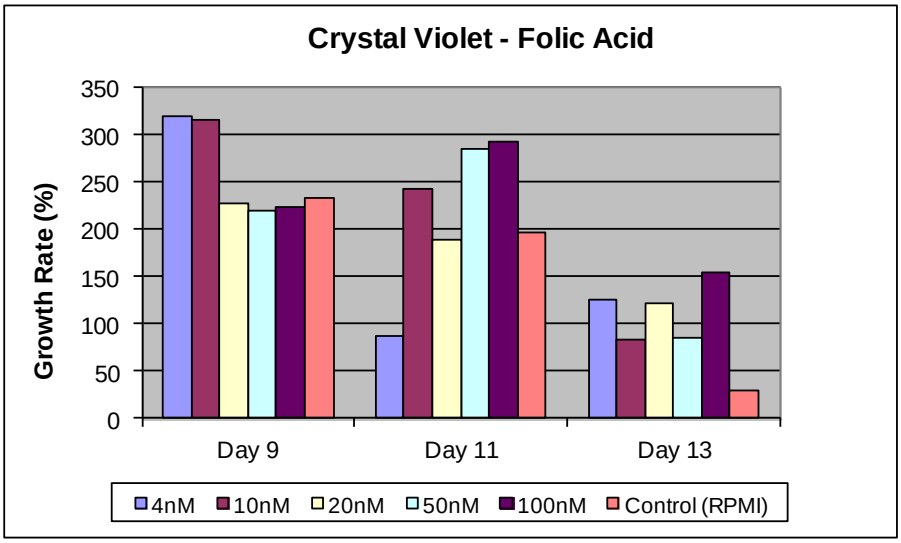
The growth rate percentage (refer to 4.4.3.2) for individual cultures was calculated (Figure 4.8) to observe the rate of cell proliferation. Calculation was done using only one value (mean value of MTT and Crystal Violet dye elution results), thus no statistical analysis was done. Based on graph plotted in figure 4.7, the growth rate percentage was lower by 0.53% - 176.23% after day 9 in all cultures, including the control (RPMI).

Meanwhile, treatment with methionine (Figure 4.5 and Figure 4.7) demonstrates that the cells are scarcely proliferating after day 11 at a methionine concentration of 20 µM and less. Figure 4.5 showed that the mitochondrial activity increased at lower rate after day 11. HT29 exposed to methionine concentration of 100 and 50 µM and control (RPMI) have significantly higher proliferation at day 13 when measured with MTT, compared to cells exposed to methionine concentration of 20 µM and less, with p value = 0.000 – 0.009.

Figure 4.7 showed that the extent of DNA replication was not further increased after day 11 if the methionine concentration was 20 μM or less. HT29 cells exposed to control (RPMI) has significantly higher proliferation at day 13 when measured with Crystal Violet dye elution, compared to cells exposed to methionine concentration of 10 μM and less, with $p = 0.009$ and 0.032 respectively. The cells appear to proliferate at a lower rate after day 9 in all cultures, including RPMI (data shown in Figure 4.9).

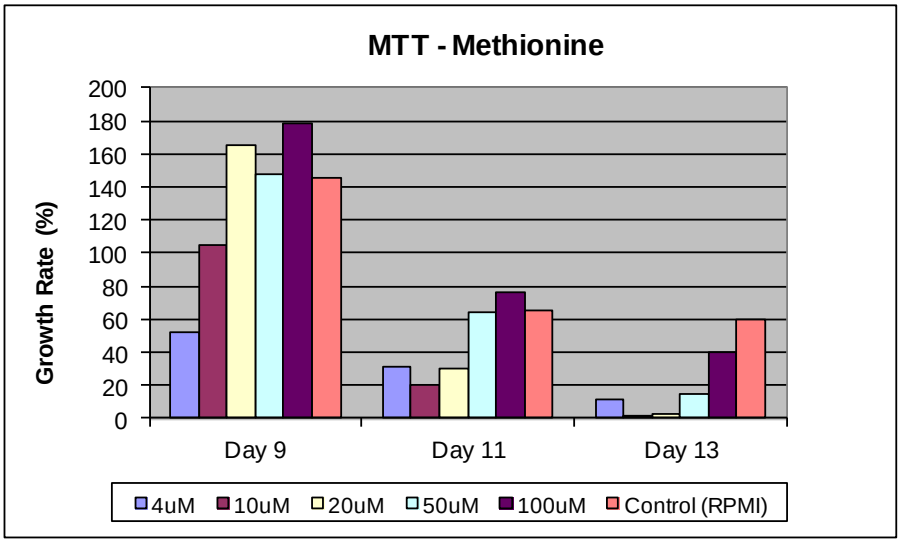


A

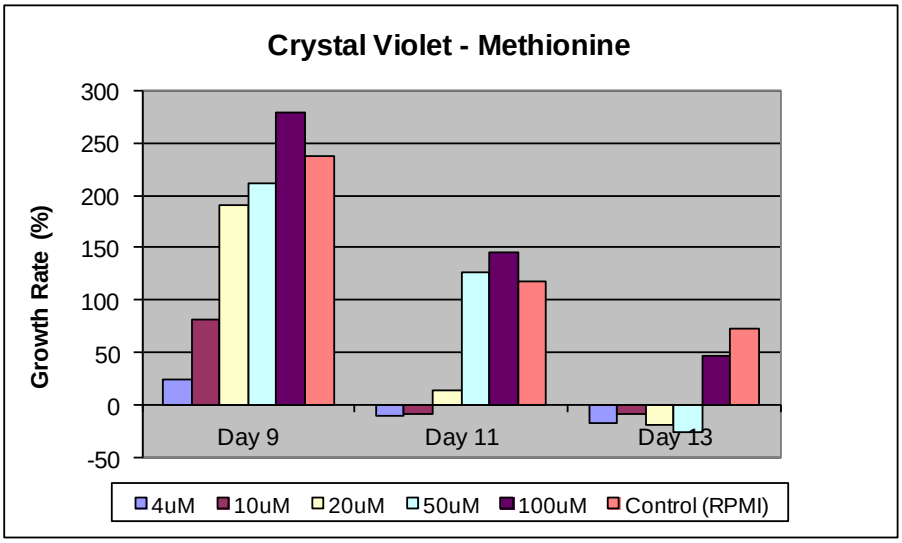


B

Figure 4.8: Growth rate of HT29 with different folic acid concentrations, measured with A: MTT assay, B: Crystal Violet dye elution assay. Control RPMI 1640 medium contained 2655 nM/L folic acid and 100.6 µM/L methionine.



A



B

Figure 4.9: Growth rate of HT29 with different methionine concentrations, measured with A: MTT assay, B: Crystal Violet dye elution assay. Control RPMI 1640 medium contained 2655 nM/L folic acid and 100.6 µM/L methionine.

4.5.2 DNA Stability in HT29 Cells

Genome stability and cytostasis of HT29 was measured with a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity; i.e. the CBMN Cyt assay [168]. DNA damage events are scored specifically in once-divided, binucleated (BN) cells and include (a) micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss, (b) nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes [129]. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells, and cytotoxicity via necrotic and/or apoptotic cell ratios [168].

4.5.2.1 Nuclear Division Index (NDI)

The effect of all folic acid and methionine concentrations investigated on nuclear divison was determined by calculating the NDI (as shown in Figure 4.10 and Figure 4.11). At day 14, the NDI of all treatments groups did not differ significantly. Folic acid deficiency did not significantly impact NDI, relative to control (RPMI) medium. A similar pattern was observed in that the highest NDI values were observed in the 20 nM folic acid and 20 µM methionine conditions. In folic acid treatment, 4 and 100 nM/L of folic acid showed lower NDI, as compared to RPMI (non-significant, p value = 1.00). In methionine treatment, it can be seen that methionine deficiency tended to reduce NDI, marginally but non-significantly (p value = 0.093 – 1.00). Post test for linear trend indicated no significant increase in the

folic acid treatment (p value = 0.7107). However, a significant increase in the linear trend for methionine treatment was observed (p value = 0.0042).

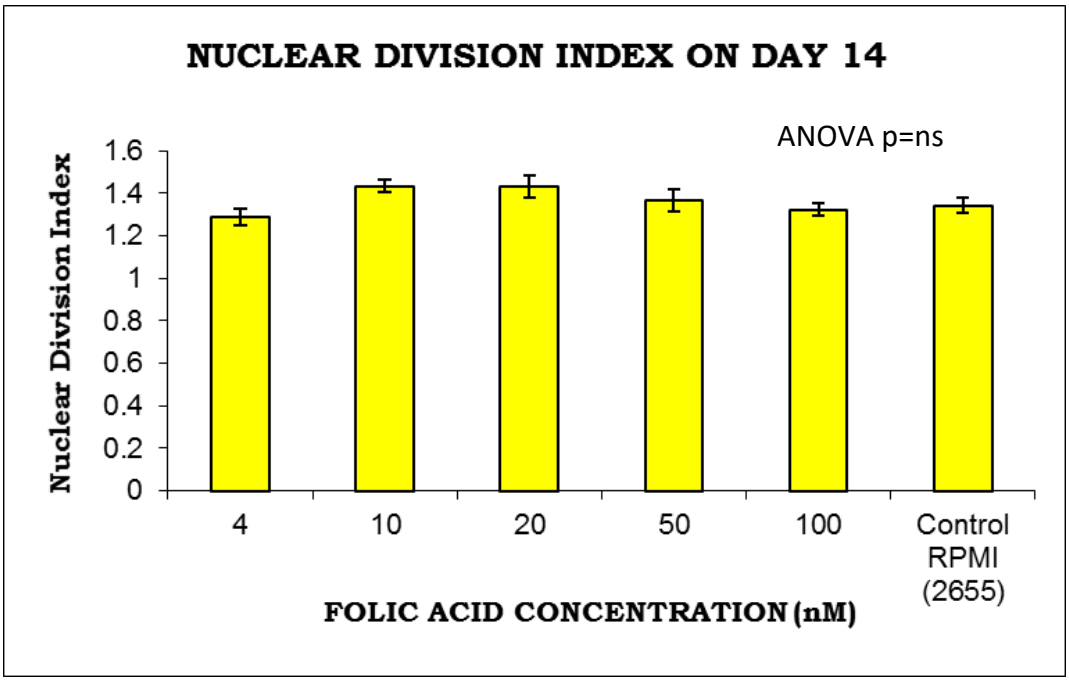


Figure 4.10: Effect of different folic acid concentration on NDI. Data expressed as mean ± SE.

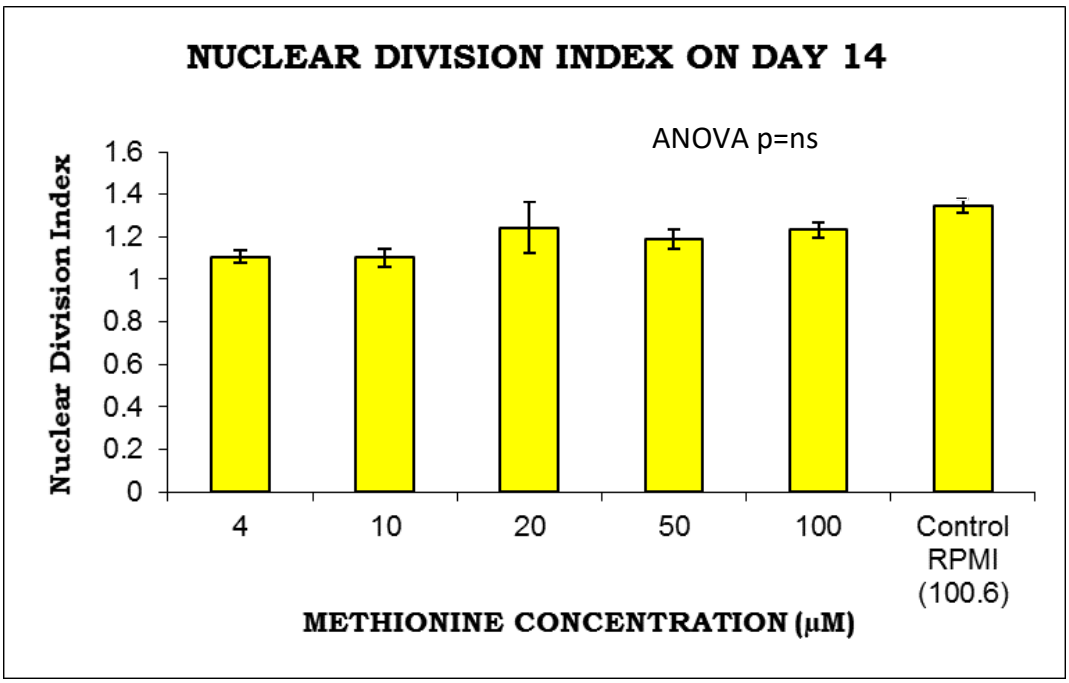


Figure 4.11: Effect of different methionine concentration on NDI. Data expressed as mean ± SE.

4.5.2.2 Apoptosis

Figure 4.12 and 4.13 represent the percentage of apoptotic cells after exposure to different folic acid and methionine concentration for 14 days. There is a significant higher apoptosis percentage in RPMI treatment that has extremely high folic acid (multiple comparison p value = 0.005 – 0.023). However there was no significant different between treatment of 4 to 100 nM folic acid (p value = 1.00). A significant increase in the linear trend for folic acid treatment was observed (p value = 0.0184), only if the high folate control data were included.

There was no significant difference of methionine treatment as compared to RPMI1640 in apoptotic cell percentage. However, there is an apparent U-shape pattern of percentage of apoptotic cells, observed in dose-dependent manner (non-significant, p value = 0.540 – 1.00). No significant linear trend (p value = 0.7107) observed.

4.5.2.3 Necrosis

Figure 4.14 and 4.15 display the percentage of necrotic cells after different treatment of folic acid and methionine concentrations for 14 days. The percentage of necrotic cells did not differ significantly among all treatment in both folic acid and methionine experiments (p value for folic acid experiment = 1.00, p value for methionine experiment = 0.86 – 1.00). No significant linear trend was observed in both folic acid (p value = 0.2345) and methionine (p value = 0.3693) experiments.

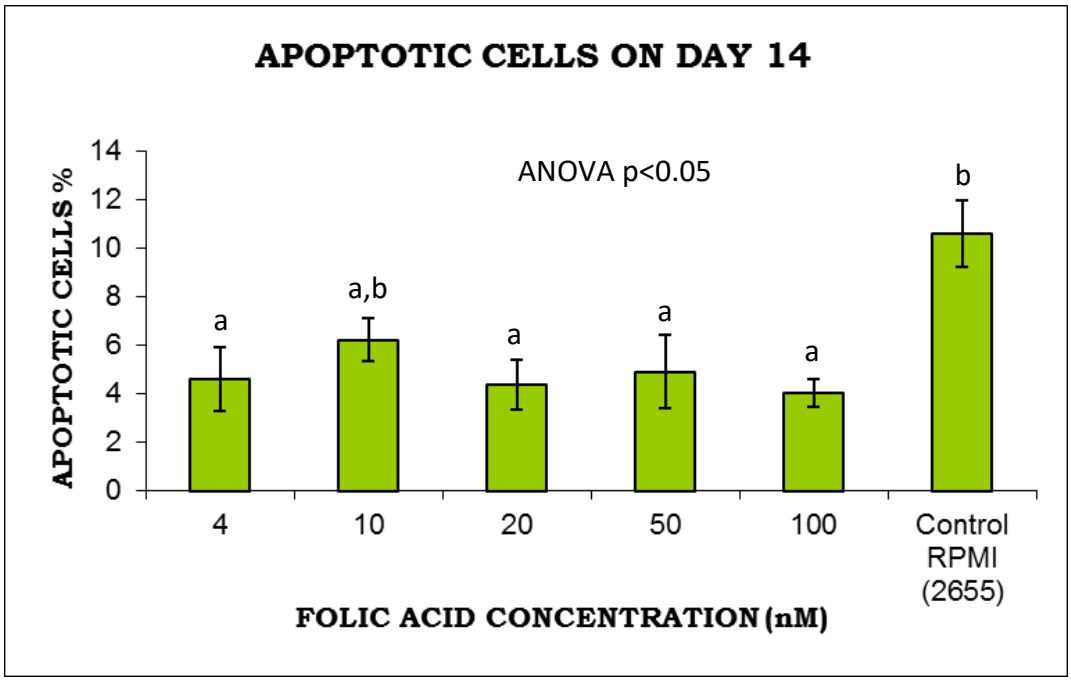


Figure 4.12: Effect of different folic acid concentration on apoptotic cell percentage. Data expressed as mean ± SE. Bars that do not share the same letter are significantly different ($p < 0.05$).

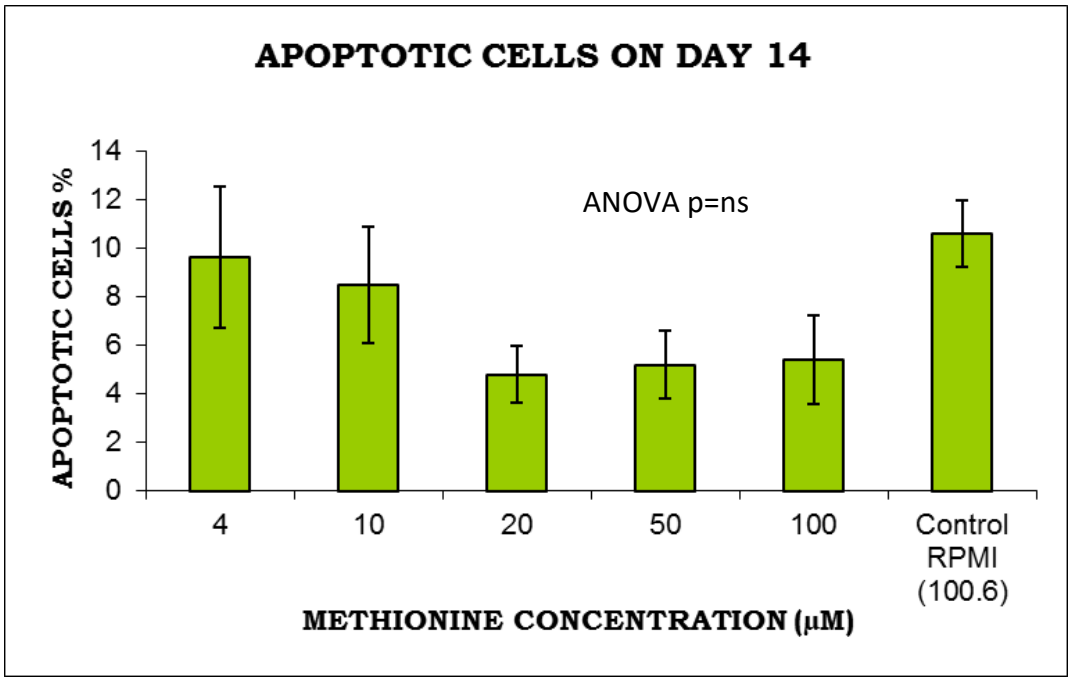


Figure 4.13: Effect of different methionine concentration on apoptotic cell percentage.

Data expressed as mean ± SE.

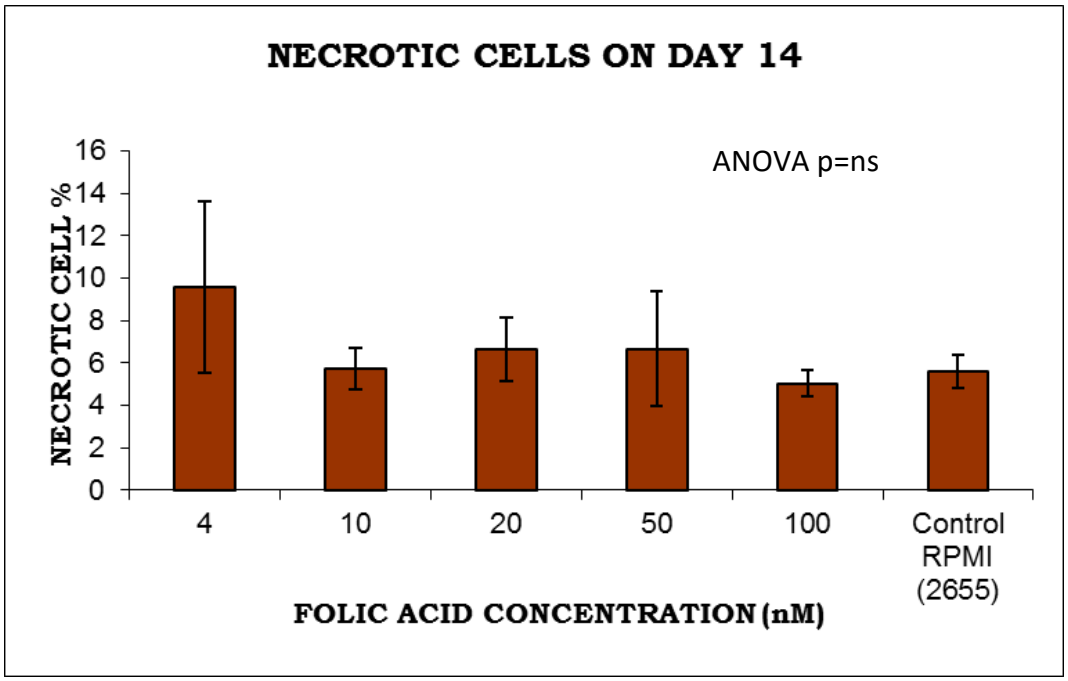


Figure 4.14: Effect of different folic acid concentration on necrotic cell percentage. Data expressed as mean ± SE.

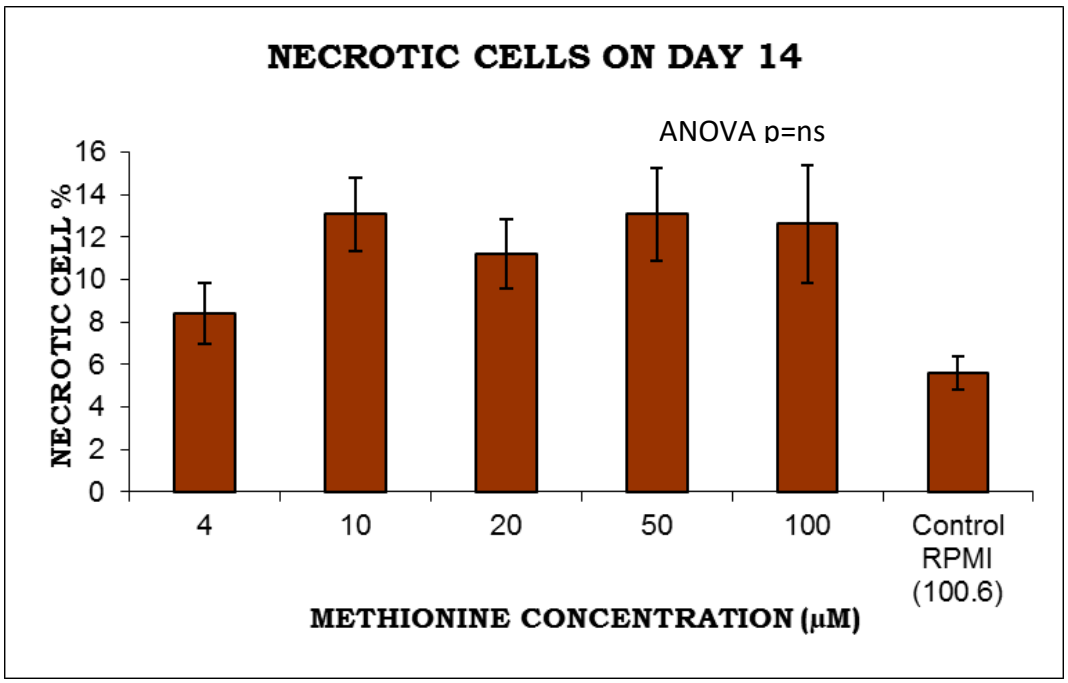


Figure 4.15: Effect of different methionine concentration on necrotic cell percentage. Data expressed as mean ± SE.

4.5.2.4 Binucleated Cells with Micronuclei

Figure 4.16 and 4.17 display the frequency of binucleated (BN) cells with micronuclei (MNi) (a biomarker of chromosome breakage and/or whole chromosome loss [168]) in HT29 after treatment of folic acid and methionine at various concentrations for 14 days. The frequency of BN cells with MNi did not differ significantly between treatments in either the folic acid or methionine experiments (p value = 1.00). However, it was observed that in the lower concentration of both treatments, the frequency of BN with MNi was highest. The frequencies in cells exposed to 4 nM folic acid and 4 µM methionine were 2.18 %, and 2.05 % respectively, compared to 1.31 % in cells exposed to control RPMI. Decreasing folic acid and methionine concentration led to an increase the frequency of BN with MNi in most of the treatment (except for 50 and 100 µM methionine) (p value = 1.00, non-significant). However, post test showed no significant linear trend was observed (p value for folic acid experiment = 0.1613, p value for methionine experiment = 0.1799).

4.5.2.5 Binucleated Cells with Nucleoplasmic Bridges

Figure 4.18 and 4.19 show the frequency of BN cell with nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions [168]. The frequency of BN cells with NPBs did not significantly differ between concentrations studied in both folic acid folic acid and methionine experiments (p value = 1.00). In these experiments, it was evident that the lowest concentration of folic acid and methionine tended to be associated with a higher frequency of BN cells with NPBs. However, no significant linear trend was observed in both folic acid (p value = 0.4534) and methionine (p value = 0.7773) experiment.

4.5.2.6 Binucleated Cells with Nuclear Buds

Figure 4.20 and 4.21 display the frequency of BN cell with nuclear buds (NBUDs) (a biomarker of elimination of amplified DNA and/or DNA repair complexes [168]) in HT29 after treatment of folic acid and methionine at various concentrations for 14 days. The frequency did not significantly differ between all treatment in both folic acid and methionine experiment (multiple comparison p value for folic acid experiment = 0.801 – 1.00, p value for methionine experiment = 1.00). However it can be observed at the lower concentration of both treatments, decreasing folic acid and methionine concentration tend to non-significantly increase the frequency of BN cells with NBUDs. Post test indicated significant linear trend in methionine experiment (p value = 0.0416). No significant linear trend was observed in folic acid experiment (p value = 0.0926).

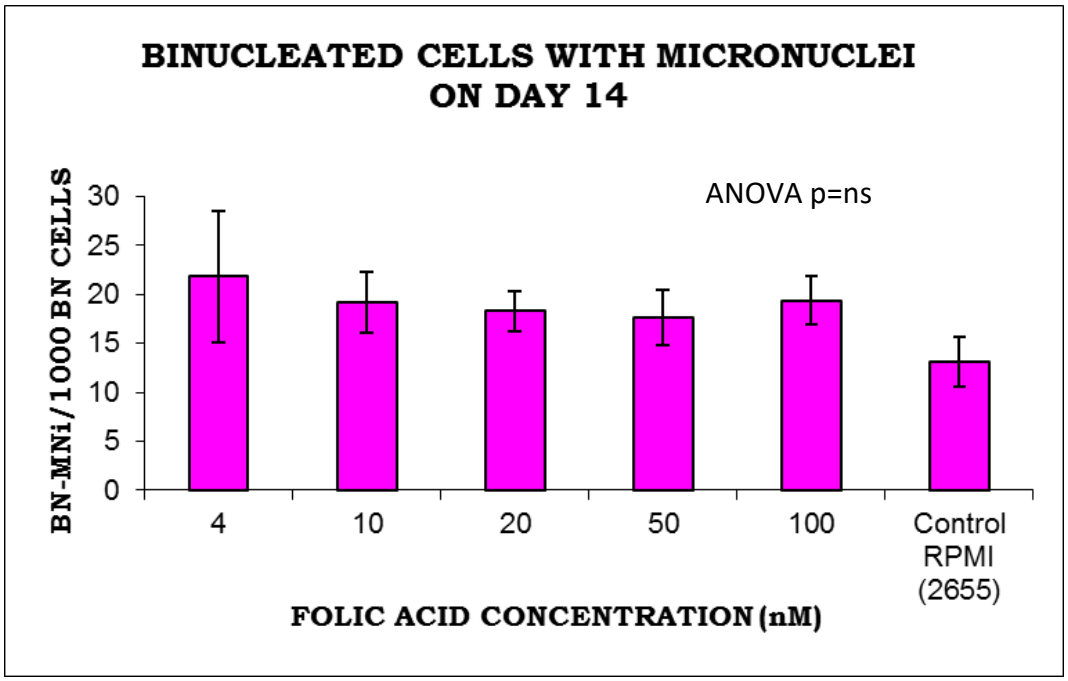


Figure 4.16: Effect of different folic acid concentration on frequency of BN cells with MNi.

Data expressed as mean ± SE.

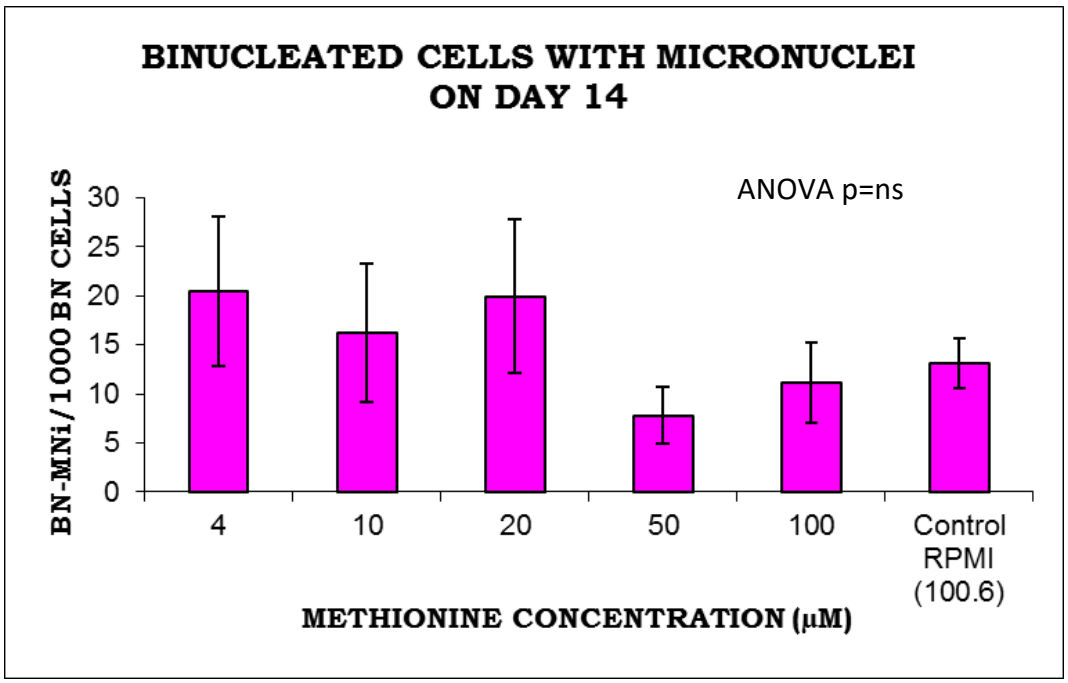


Figure 4.17: Effect of different methionine concentration on frequency of BN cells with

MNi. Data expressed as mean ± SE.

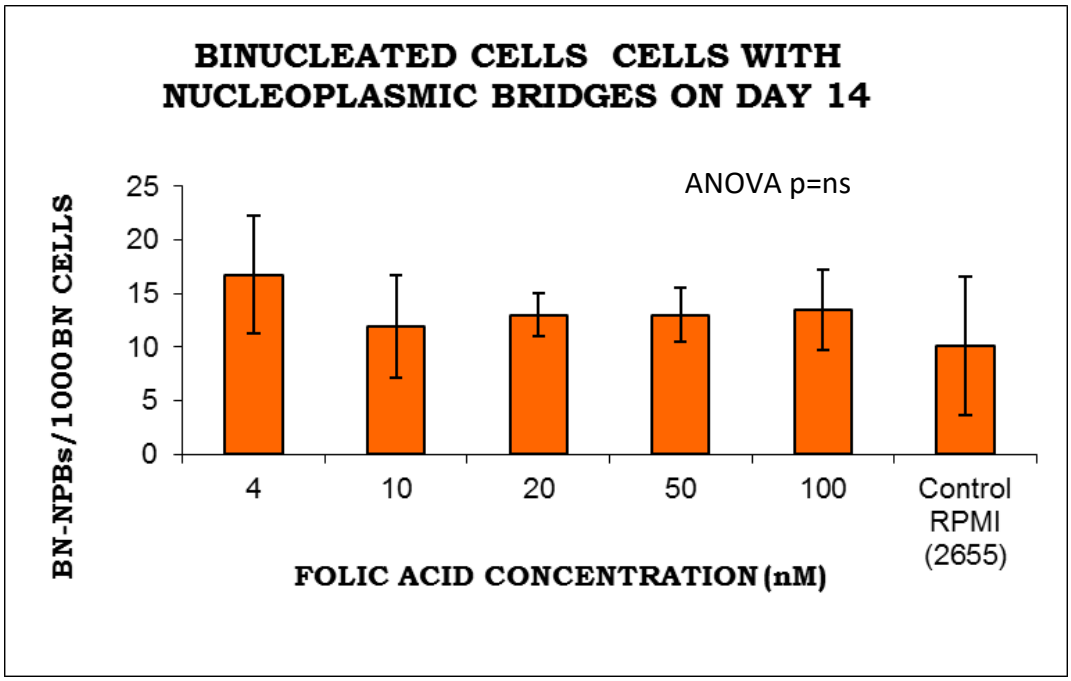


Figure 4.18: Effect of different folic acid concentration on frequency of BN cells with NPBs.

Data expressed as mean ± SE.

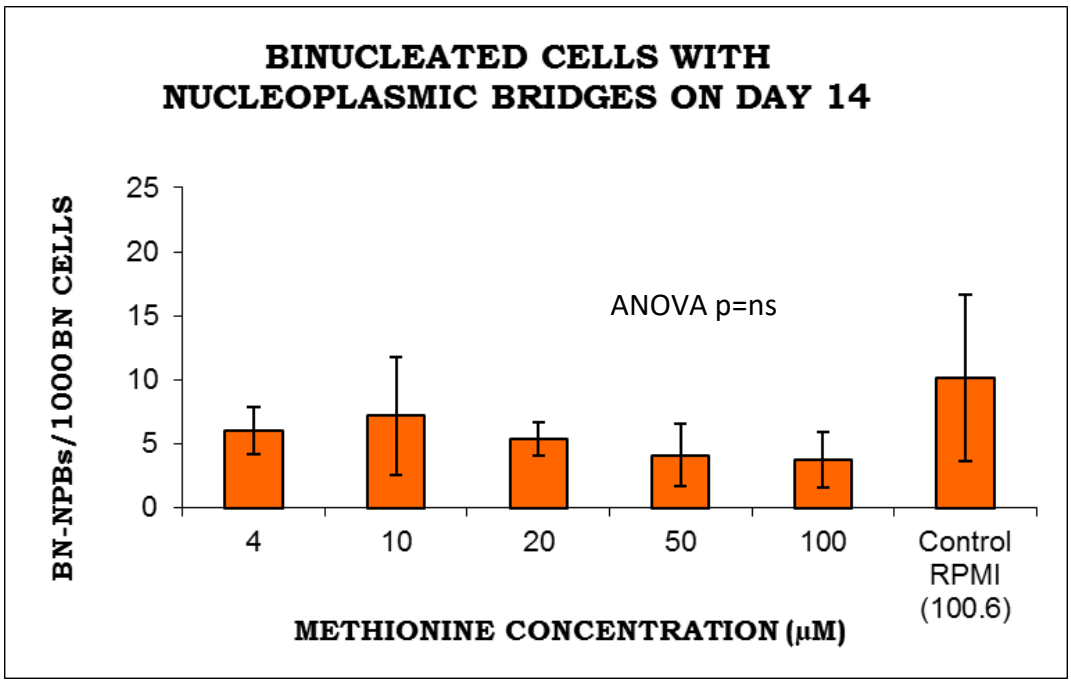


Figure 4.19: Effect of different methionine concentration on frequency of BN cells with

NPBs. Data expressed as mean ± SE.

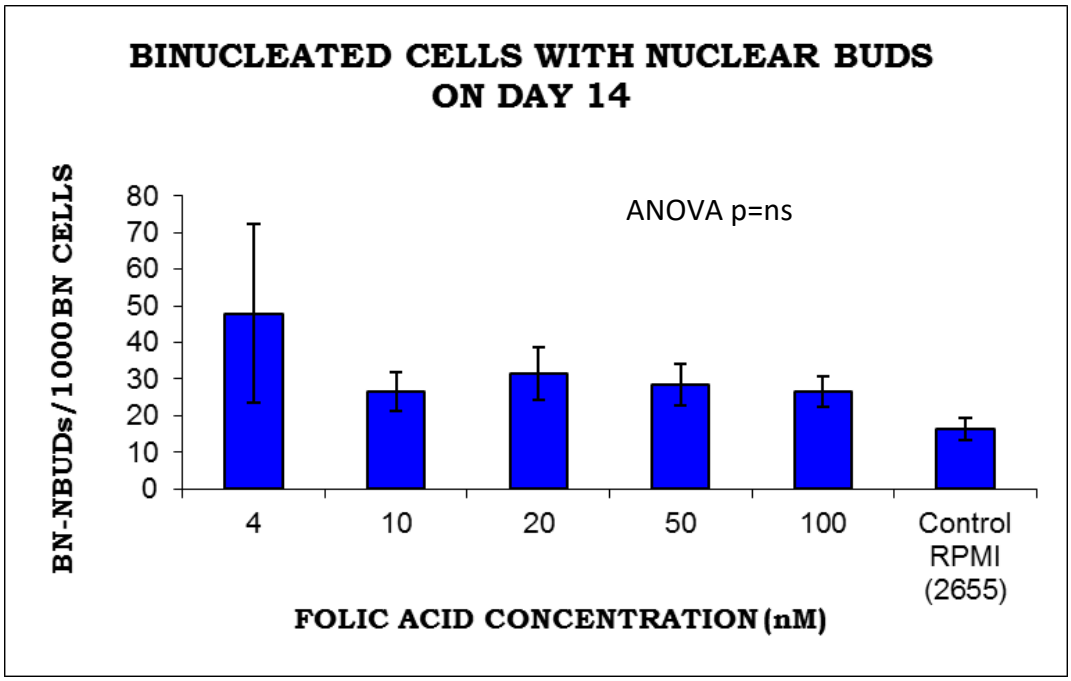


Figure 4.20: Effect of different folic acid concentration on frequency of binucleated cells with nuclear buds. Data expressed as mean ± SE.

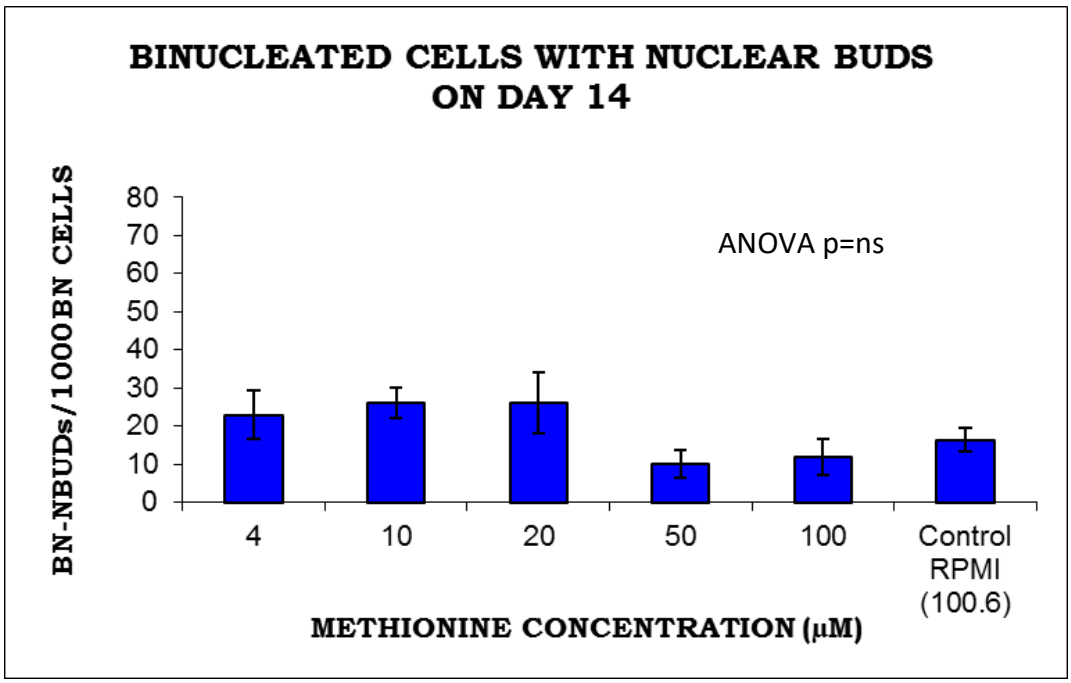


Figure 4.21: Effect of different methionine concentration on frequency of binucleated cells with nuclear buds. Data expressed as mean ± SE.

4.6 Discussion

Current approaches to control cancers include the use of cytotoxic drugs that either inhibit folate metabolism and DNA synthesis such as Methotrexate or other drugs that damage DNA by causing DNA strand breaks, DNA crosslink or DNA adducts [171]. An alternative approach is to restrict nutrients required for DNA synthesis, DNA repair and cell proliferation, such as folate and methionine [27]. The reason of using HT29 was to understand whether restriction of folic acid or methionine reduces the proliferative growth of HT29 cancer cells and to establish whether growth inhibition was due to increases in DNA damage. The results of the study showed that DNA damage was not significantly increase in the CBMN Cyt biomarkers (frequencies of binucleated cell with micronuclei, nucleoplasmic bridges, and nuclear buds) suggesting that there must be other reasons why cell proliferation and cell viability declined with folic acid and methionine deficiency.

Folate and methionine are important cofactors in a large number of metabolic processes, such as nucleotide synthesis, interconversion of amino acids, and methylation [172, 173]. In this study, HT29 cell proliferation decreased when folic acid and methionine were decreased, in a dose-dependent manner. Cell proliferation in the standard RPMI1640 condition was significantly higher than in all other folic acid conditions ($p<0.05$ for each). Methionine deficiency (less than 20 μM) significantly inhibited cell proliferation ($p<0.05$). These data are in agreement with previous investigations that show similar inhibiting effect of HT29 cell growth with depletion of folate and methionine [156-158, 174, 175]. In Pellis

et al. (2008) [156] higher concentration of folate resulted in higher cell growth, and higher concentration of intracellular 5'-methyltetrahydrofolate, SAM and SAH.

Insufficiency of folate reduced DNA synthesis (as tested by crystal violet assay), and lowered the proliferation rate of cells. In the methionine cycle, previous evidence has shown a lack of methionine supply leads to impaired polyamine production [176], a mechanism potentially underlying the reduced growth rate observed here. It is also suggested that higher cell proliferation indicates higher intracellular ATP level. This is based on physiological observation that demonstrate an effect of folate on ATP content and MTT conversion [156]. This is relevant due to the function of one-carbon metabolism in the synthesis of formate, glycine and f-met-tRNA in mitochondria [177], in which folate and methionine has direct role in the one-carbon metabolism. These mechanisms may feasibly explain the observation in the current study.

In the investigation of DNA stability in HT29 exposed to different folic acid and methionine concentration, it is shown that higher concentration of folate (as observed in control RPMI) induces apoptosis ($p < 0.05$). Perhaps folate excess may protect against cancer cell growth by induction of apoptosis. This is in agreement with similar pattern observed in Pellis et al. (2008) [156], showing that higher folic acid concentration induces increased apoptosis in HT29, compared to lower folic acid concentration. Methionine had no significant effect on apoptosis in this study. However, previous studies have shown that selenomethionine treatment induced apoptosis in HT29 and A549 lung cancer cells [178],

and SAM induced apoptosis in PC12 cell line [179]. Whether higher methionine concentration caused an increase in SAM concentration was not investigated in the present study, but may be interesting to explore.

Folic acid and methionine restriction did not alter other parameters of DNA stability (necrosis, and the frequencies of binucleated cell with micronuclei, nucleoplasmic bridges, and nuclear buds). This may be because of the lack of sensitivity to DNA damage induced in HT29 cells after a long period of incubation (14 days). Crystal violet dye elution results suggested that the DNA replication in HT29 cells is decreased after day 9 of incubation.

One mechanism of folate transport into the cells is the high affinity folate receptor [180, 181]. In the present study, it is speculated that the resistance of HT29 to DNA damage when folic acid is deficient might be due to overexpression of folate receptor in HT29 cells [182-184]. The expression of folate receptor in HT29 cells is 1.5 fold higher compared to Caco-2 colorectal cancer cells [183]. Overexpression of folate receptor α 1 (FRα1) allows HT29 cells to adapt to high concentration of antifolate drug, methotrexate [184].

In a study by Rodriguez et al. (2015), HT29 cultured in 20 nM folic acid had higher percentage of cell surface FRα1 expression, compared to higher folic acid concentration at 400 nM. However, there was no difference observed in the total amount of cell surface and internalized FRα1 [175]. Another study by Li et al. (2011) observed a slight increase in fluorescence intensity viewed under fluorescent microscope, indicating cellular uptake of

folic acid-chitosan nanoparticles, in HT29 cultured in folic acid free media. This demonstrates high binding affinity of overexpressed folic acid receptor in HT29 cells [182].

In conclusion, restriction of folic acid or methionine prevents excessive (rapid) cellular proliferation, and relative to high folate, the lower folate concentration reduces apoptosis in HT29 cell lines. It is apparent that HT29 cells may be resistant to genome instability induced by folic acid or methionine deficiency under the experimental conditions reported for this study, and this may be due to upregulation of folate receptor. With regard to cancer, the findings in the *in vitro* study suggest that restricting folate or methionine slow the growth of colon cancer cells without increasing chromosomal instability or DNA damage.

A weakness of this study was that results were restricted to HT29 cells and it remains unknown whether the same effects would apply to other colon cancer cell lines such as SW620, HCT 116 and Caco-2. Future studies should be aimed at comparing the effects in different colon cancer cell lines to determine the extent of variation in response between them. It is also important to verify the observations made and to test the possible mechanisms such as amplification of the folate receptor.

Chapter 5:

In Vitro Studies – II

The Effect of Folic Acid and Methionine on Telomere Length and DNA Methylation in HT29

5.1 Objective

The aim of this study was to investigate the effect of folic acid and methionine depletion on genomic instability measured via telomere length and DNA methylation in HT29 cells.

5.2 Hypotheses

3. Folic acid or methionine deficiency are associated with increased telomere length in HT29 cells.
4. Folic acid or methionine deficiency result in decreased DNA methylation in HT29 cells.

5.3 Introduction

Genetic instability arising from dysfunctional telomeres is a potential cancer predisposing factor. Telomeres consist of long $(TTAGGG)_n$ nucleotide repeats, together with an associated protein complex [185]. These nucleoprotein structures cap the ends of linear chromosomes, which are essential for maintaining chromosomal stability. They protect the

end of the chromosome from end-to-end fusions, recombination, and shortening [186, 187]. Excessively short telomeres have been linked with higher risk of several types of cancers [188].

Telomerase activity, and recombination, are the two major mechanisms involved in telomere length maintenance [189, 190]. Telomeres are synthesized by telomerase at the chromosome ends, regulating the length of telomere repeats [191]. However, in a small percentage of cancerous cells telomeres are maintained by an "alternative lengthening of telomeres", or ALT, mechanism[192], without the presence of telomerase (telomerase-negative) [193, 194]. The ALT mechanism is based on homologous recombination of telomere sequences [189]. HT29 is a telomerase-positive (ALT-negative) cell line [195, 196]. Previous work by Zhao et al. (2010) looking at possible relationship between hRAD21 and the ALT pathway in ALT-positive and ALT-negative cell lines proved that HT29 maintains telomere length by the activation of telomerase [195].

Growing evidence has also linked epigenetic regulation with telomere length maintenance [197-200]. Epigenetic modification, in the form of DNA methylation and/or histone modification, are suggested mechanisms that have important function in telomere length homeostasis [197, 201]. DNA methylation is suggested to have negative regulatory effect on telomere length [197, 198, 202]. Gaddala et al. (2012) [202] reported inverse correlations between telomere length and subtelomeric, LINE-1, or pericentromeric

methylation in human dyskeratosis congenita cells. This is in agreement with a study by Vera et al. (2008) [198] performed using a panel of human cancer cell lines.

DNA methylation plays a vital role in maintaining cellular function. Dysfunctions in DNA methylation processes may contribute to the development of cancer [203]. Aberrant methylation of DNA is frequently found in tumor cells [204]. Global DNA hypomethylation can result in chromosome instability and abnormal expression of oncogenes [199, 205]. Meanwhile, hypermethylation has been associated with reduced expression of tumor suppressor genes [206].

Folate and methionine have been shown to have effects on DNA methylation [207, 208]. This is based on their roles in the biochemical pathways involved in DNA methylation, which influences the supply of methyl groups required by DNA methyltransferases to perform their function [209]. As discussed previously (refer to Chapter 2.2.1 and Figure 2.1) folate plays an important role in one-carbon metabolism, by which a carbon unit from serine or glycine is transferred to tetrahydrofolate to form 5,10-methylenetetrahydrofolate [210]. 5,10-methylenetetrahydrofolate is then reduced to 5-methyltetrahydrofolate and used to methylate homocysteine to form methionine [211]. Methionine is converted to SAM, that donates its labile methyl groups to methylation reactions, including the methylation of DNA, RNA, and protein [172, 211].

There is evidence that telomere length is epigenetically regulated by changes to DNA methylation status, and that altered nutritional status may contribute to this effect [212-214]. The hypomethylating effect of folate deficiency has been demonstrated to result in significant telomere elongation in human WIL2-NS cells [199]. Paul et al. (2009) have also shown that folate deficiency may impact on telomere length (both lengthening and shortening) in lymphocytes *in vivo* [212]. The effect of nutrient deficiency on telomeres in HT29 cells had not previously been examined. Thus, this study was carried out to investigate the effects of folic acid and methionine deficiency in the human colon adenocarcinoma cell line, HT29. HT29 cells has been widely used in the investigation of related intestinal function and differentiation, as it has been shown to be able to express the differentiation features which are characteristic of mature intestinal cells, such as enterocytes or mucus cells [215].

5.4 Material and Methods

5.4.1 Study Design

To examine the effect of folic acid and methionine depletion on telomere length and DNA methylation in HT29 cells, experiments were carried out following the study design as shown in Figure 5.1. The concentrations of folic acid and methionine used are the same as those detailed for *in vitro* studies – I (refer to Chapter 4.4.2).

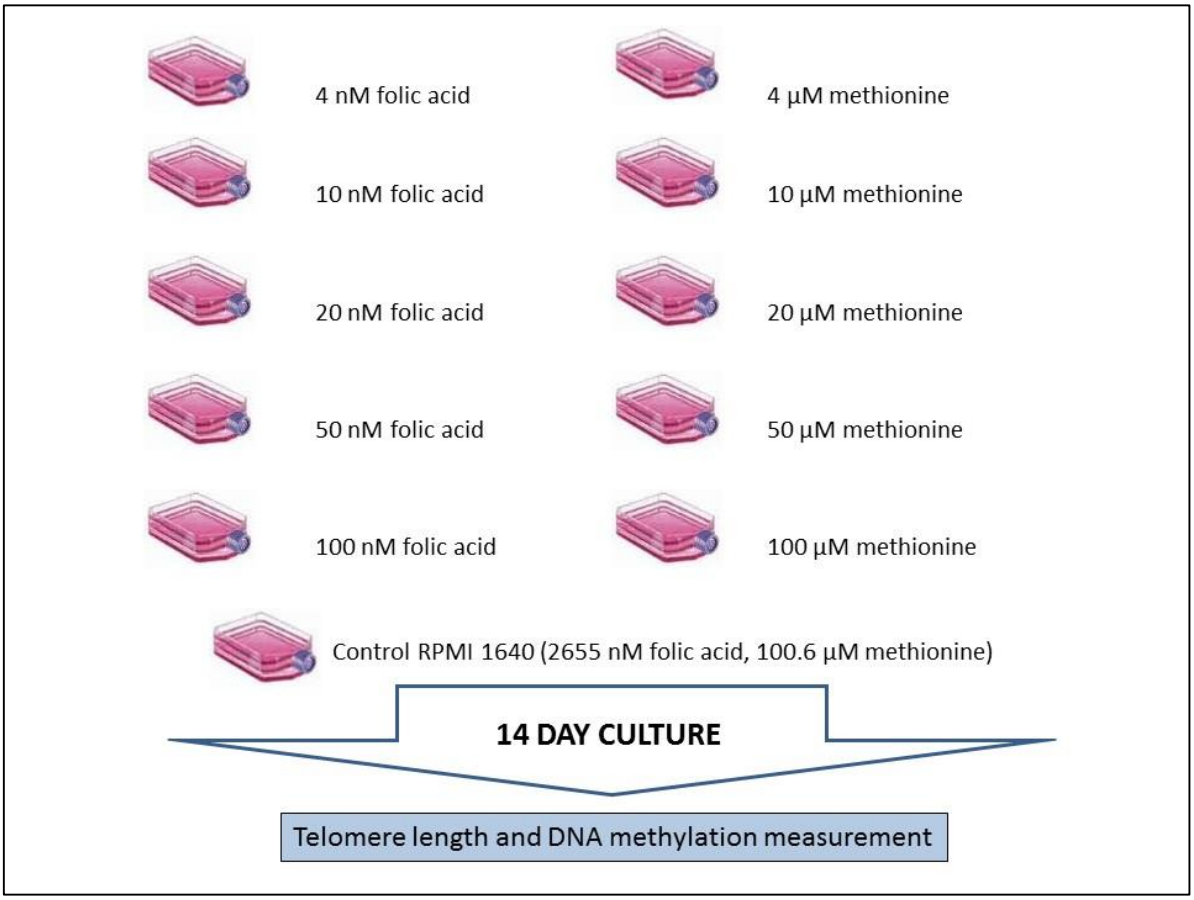


Figure 5.1: Study design of experiment to investigate the effect of folic acid and methionine depletion on telomere length and DNA methylation in HT29 cells.

5.4.2 14 Day Culture of HT29 Cells

HT29 cells (ATCC HTB-38, Manassas, VA, USA) were cultured in 75cm^2 culture flasks (Thermo Fisher Scientific, NY, USA), and grown in normal RPMI 1640 (Sigma, St. Louis, MO, USA) medium and medium containing different concentrations of folic acid and methionine (preparation as described in 4.4.2) for 14 days:

- Control: RPMI 1640 medium (containing 2655 nM/L folic acid, 100.6 µM/L methionine)
- Folic acid experiment: 4, 10, 20, 50 & 100 nM/L folic acid (with constant level of methionine at 100.6 µM/L).
- Methionine experiment: 4, 10, 20, 50 & 100 µM/L methionine (with constant level of folate at 2655 nM/L).

All media were supplemented with 5% (v/v) foetal bovine serum (FBS) (Thermo Trace, Australia), 1% 1 mM L-glutamine (Sigma, USA) and 1% (v/v) penicillin (5000 IU/ml)/streptomycin (5 mg/mL) (Sigma, USA). Cells were maintained in incubator at 37°C with 5 % CO_2 and 100 % relative humidity. Culture medium was replaced (100%) every other day. Cells were split at 80 - 90 % confluence. On day 14, cells were harvested and resuspended in 200 µL PBS for DNA isolation.

5.4.3 DNA Isolation

Isolation of DNA from HT29 cells harvested was carried out using DNeasy Blood and Tissue Kit 250 (Qiagen, Melbourne, Australia). Manufacturer's protocol was followed with

minor modification as mentioned in [216]; ie. to minimize DNA oxidative damage, all solutions used were purged with nitrogen and supplemented with 50 µM phenyl-tert-butyl nitrone (Sigma, St. Louis, MO, USA), prior to DNA isolation process [217].

200 µL of HT29 cells suspension were placed in microcentrifuge tube, then added with 20 µL proteinase K 600 mAU/ml) and 180 µl Buffer AL (tissue and cell lysis buffer). The mixture was then incubated at 37°C for 6 hours to completely lyse the cells. 200 µL ethanol was added and incubated for 3 minutes at room temperature. The mixture was pipetted into spin column, and centrifuged at 8000 rpm for 1 minute. The flow through was discarded. 500 µL Buffer AW1 (wash buffer) was added to the coulmn, and centrifuged at 8000 rpm for 1 minute. The flow through was discarded and 500 µL Buffer AW2 (wash buffer) was added to the column. The mixture in the spin column was centrifuged at 14000 rpm for 3 minute, flow through discarded, and the column placed in a new microcentrifuge tube. 200 µL Buffer AE (elution buffer) was added directly in to the column, incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute. The eluate was then pipetted back into the spin column, incubated at room temperature for 1 minute and centrifuged again at 8000 rpm for 2 minutes. The final volume of approximately 200 µL of DNA concentrate was obtained. The concentration of DNA for each sample was determined with 260/280 nm measurement using NanoDrop (Biolab, ND 1000). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. Two readings were recorded for each sample and averaged.

5.4.4 Telomere Length Assay

5.4.4.1 Quantitative Real-time Polymerase Chain Reaction (qPCR) for Telomere Length

Absolute telomere length of all DNA samples was measured with qPCR following the method described by O'Callaghan et al [216]. All samples were run on an ABI 7300 Sequence Detection System with the SDS Ver. 1.9 software (Applied Biosystems, Foster City, CA).

16 µL master mix solution was pipetted into each well of a 96-well PCR plate, consisting of 10 µL SYBR green master mix (Applied Biosystems, Life Technologies, California), 4 µL H_20, 1 µL of primer (telomere-fwd (2 µM)), and 1 µL of Primer (telomere-rev (2 µM)). 4 µL of DNA (5 ng/µL DNA) was then added. For negative control, 4 µL of H_20 was added. For positive control, 4 µl of a long telomere positive control DNA (cell line 1301; accession number 01051619, European Collection of Cell Cultures, UK EQUIPMENT) was used. Telomere 84mer standard, $(TTAGGG)_{14}$ (TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA GGGTTAGGGTTAGGGTTAGGG) was used as standard. The PCR plate was then sealed, centrifuged, and run in the qPCR machine. Cycling conditions were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by a dissociation curve.

5.4.4.2 qPCR for 36B4 Single Copy Gene (SCG)

36B4 single copy gene (SCG) was measured with qPCR following the method described in [216]. 36B4 is routinely used for human samples to determine number of genome copies in each sample [216]. A standard curve was generated by performing a serial dilution of $10^3 – 10^8$. Cycle threshold (Cq) values obtained from SCG were used to calculate the number of genome copies per reaction. [216].

16 µL master mix solution was pipetted into each well of a 96-well PCR plate, containing 10 µL SYBR green master mix (Applied Biosystems, Life Technologies, California, USA), 4 µL H_20, 1 µL of 36B4 forward primer (1:10 stock), and 1 µL of 36B4 reverse primer (1:10 stock). 4 µL of DNA (5 ng/µL DNA) was then added. For negative control, 4 µL of H_20 was added. For positive control, 4 µL of a 1301 DNA (European Collection of Cell Cultures, UK EQUIPMENT) was used. The PCR plate was then covered with plastic adhesive, centrifuged, and run in the qPCR machine. Cycling conditions were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by a dissociation curve.

5.4.4.3 Calculation of Telomere Length

Values of kb/reaction for telomere and genome copies/reaction for SCG were recorded. The kb/reaction value was then used to calculate total telomere length in kb per human diploid genome. The telomere kb per reaction value was divided by diploid genome

copy number (calculated from the 36B4 Cq and standard curve) to give a total telomere length in kb per human diploid genome.

5.4.6 DNA Methylation Assay

Global DNA methylation of HT29 cells after 14 days in culture was measured using the MethylFlash Methylated DNA Quantification Kit (Colorimetric) (Epigentek, USA), following the manufacturer's recommended protocol. In this assay, DNA is bound to strip wells that are specifically treated to have high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibodies and then quantified colorimetrically by reading the OD in a microplate spectrophotometer. The amount of methylated DNA is proportional to the OD measured.

80µL of ME2 (binding solution) was pipetted to each well. 1µL ME3 (negative control, unmethylated polynucleotide containing 50% of 5-cytosine, 20 µg/mL), 1µL positive control (methylated polynucleotide containing 50% of 5-methylcytosine), and 2µL of sample DNA (at 100ng/µL) were added to designated wells. Plates were sealed and incubated at 37°C for 90 minutes, then washed with 150µL of diluted ME1 (10X wash buffer) 3 times.

50µL of diluted ME5 (capture antibody, 1000 µg/mL) was added to each well, sealed and incubated at room temperature for 60 minutes. Solution was then removed from wells and washed with 150µL of diluted ME1 for 3 times. Then, 50µL of diluted ME6 (detection antibody, 400 µg/mL) was added, sealed and incubated at room temperature for 30

minutes. Solution was then removed from wells and washed with 150µL of diluted ME1 for 4 times. 50µL of diluted ME7 (enhancer solution) was added, sealed and incubated at room temperature for 30 minutes. Solution was then removed from wells and washed with 150µL of diluted ME1 5 times.

100µL ME8 (developer solution) was added to each well and incubated at room temp for 1-10 minutes away from light to monitor colour changes; the depth of the blue colour indicates the degree of DNA methylation. 50 µL ME9 (stop solution) was then added to each well to stop enzyme reaction when colour in the positive controls wells turns medium blue. OD was read at 450nm within 2-15 minutes with ELISA microplate reader (SpectraMax 250, Molecular Devices, CA, USA). Results were expressed as 5-methylcytosine percentage based on the following formulas:

5-methylcytosine (ng) =

$$\frac{\text{Sample OD} - \text{ME3 OD}}{\text{Slope of standard curve X 2}}$$

5-methylcytosine % =

$$\frac{\text{5-methylcytosine (ng)}}{\text{Amount of input sample DNA (ng)}} \times 100$$

5.4.7 Statistical Analyses

All data were analysed with IBM SPSS Statistic 21. Treatment effects were analysed by repeated measure One Way ANOVA with Post Hoc Multiple Comparison Bonferroni Test to determine significance of difference between treatments. Bivariate Pearson Correlation

test was carried out to determine the relationship between telomere length and DNA methylation. Post test for linear trend was performed in GraphPad Prism 7. Significance was accepted at $p < 0.05$.

5.5 Results

5.5.1 Telomere Length

Figure 5.2 represents the values of telomere length (in kb per human diploid genome) in HT29 cultured in different folic acid concentrations for 14 days. Post Hoc Test (Multiple Comparison Bonferroni) shows that HT29 cells cultured in folic acid concentrations in the range of 4 to 50 nM had significantly longer telomeres after 14 days culture, compared to control cells grown in normal RPMI medium which has a folic acid concentration of 2655 nM (multiple comparison p value = 0.00 – 0.026) (refer to Table 5.1). There was no significant difference of telomere length in cells cultured in 100nM folic acid and control RPMI. Cells grown at 20 nM folic acid had the longest telomeres.

It was also found that low methionine concentrations (in the range of 4 to 50 μM) increase telomere length in HT29 cells (refer to Figure 5.3) cultured for 14 days, compared to cells grown in control (RPMI) (p value = 0.00) (refer to Table 5.2), which has a methionine concentration of 100.6 μM. Post test for linear trend indicated a significant decrease in telomere length in both folic acid and methionine experiments, across the treatment from low to high concentration (p values < 0.0001 for both experiments).

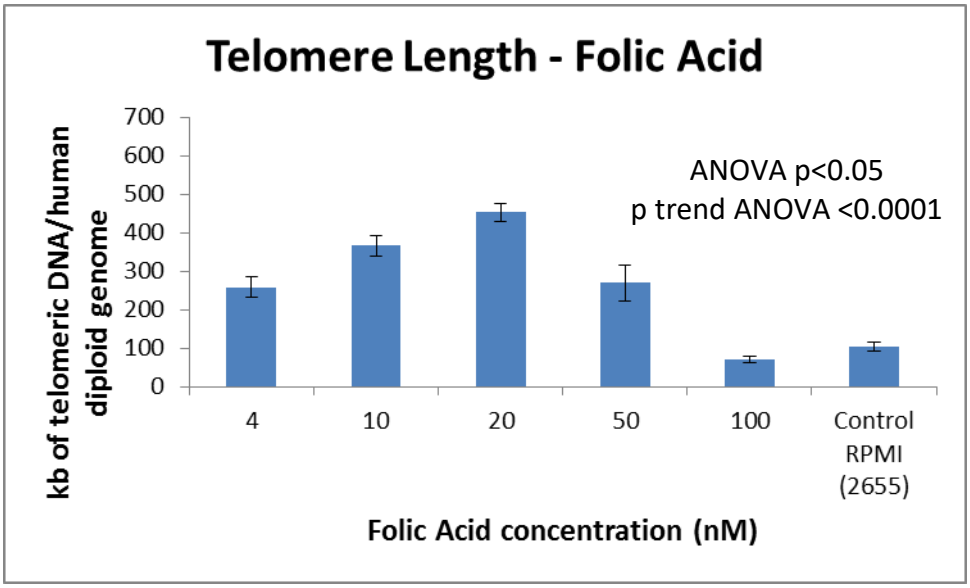


Figure 5.2: Effect of folic acid concentrations on telomere length in HT29 cells cultured for 14 days. Data expressed as mean ± SE of triplicates. Significance of difference is tabulated in Table 5.1.

Table 5.1: Post Hoc comparison using Multiple Comparison Bonferroni Test to determine significance of difference between folic acid concentrations on telomere length in HT29 cells. Data shown as p value. * shows the mean difference is significant at $p<0.05$ level. NA = not applicable.

Folic acid concentration (nM)	**4**	**10**	**20**	**50**	**100**	**Control RPMI (2655)**
4	NA	0.219	*0.004	1.000	*0.005	*0.026
10		NA	0.606	0.397	*0.003	*0.000
20			NA	*0.006	*0.000	*0.000
50				NA	*0.000	*0.015
100					NA	1.000
Control RPMI (2655)						NA

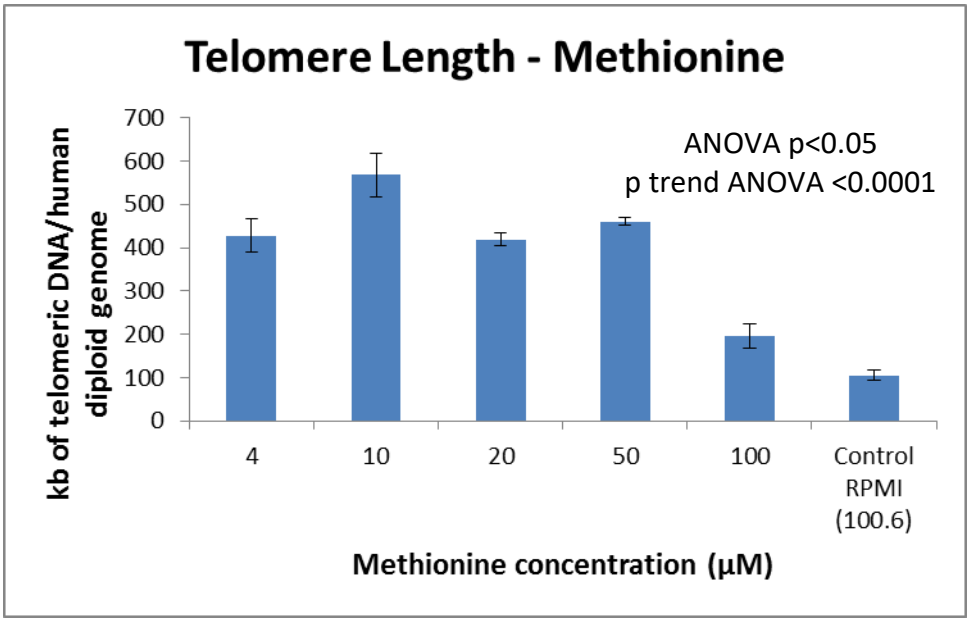


Figure 5.3: Effect of methionine concentrations on telomere length in HT29 cells cultured for 14 days. Data expressed as mean ± SE of triplicates. Significance of difference is tabulated in Table 5.2.

Table 5.2: Post Hoc comparison using Multiple Comparison Bonferroni Test to determine significance of difference between methionine concentrations on telomere length in HT29 cells. Data shown as p value. * shows the mean difference is significant at $p<0.05$ level. NA = not applicable.

Methionine concentration (μM)	**4**	**10**	**20**	**50**	**100**	**Control RPMI (100.6)**
4	NA	*0.008	1.000	1.000	*0.002	*0.000
10		NA	0.055	0.337	*0.000	*0.000
20			NA	1.000	*0.003	*0.000
50				NA	*0.001	*0.000
100					NA	0.726
Control RPMI (100.6)						NA

5.5.2 DNA Methylation

Figure 5.4 and 5.5 display the values of global DNA methylation (5-methylcytosine percentage) in HT29 treated with different folic acid and methionine concentrations for 14 days. Post Hoc Test (Multiple Comparison Bonferroni) showed that global DNA methylation did not change significantly with folic acid or methionine concentration after 14 days culture, compared to control (RPMI) (multiple comparison p value = 0.134 – 1.000 for folic acid treatment, p value = 0.188 – 1.000 for methionine treatment) (refer to Table 5.3 and 5.4). No significant linear trend was found in folic acid experiment (post test p value = 0.350). In the methionine experiment, post test for linear trend showed a significant decrease across the treatment groups, from low to high concentration (p value = 0.010).

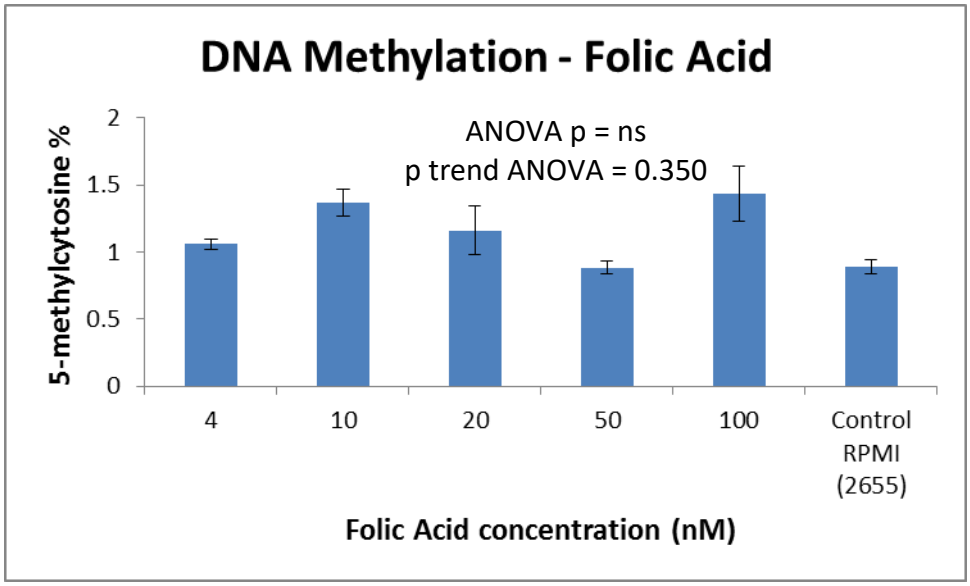


Figure 5.4: Effect of folic acid concentrations on DNA methylation in HT29 cells cultured for 14 days. Data expressed as mean ± SE of triplicates.

Table 5.3: Post Hoc comparison using Multiple Comparison Bonferroni Test to determine significance of difference between folic acid concentrations on DNA methylation in HT29 cells. Data shown as p value. NA = not applicable.

Folic acid concentration (nM)	**4**	**10**	**20**	**50**	**100**	**Control RPMI (2655)**
4	NA	1.000	1.000	1.000	0.759	1.000
10		NA	1.000	0.136	1.000	0.154
20			NA	1.000	1.000	1.000
50				NA	0.120	1.000
100					NA	0.134
Control RPMI (2655)						NA

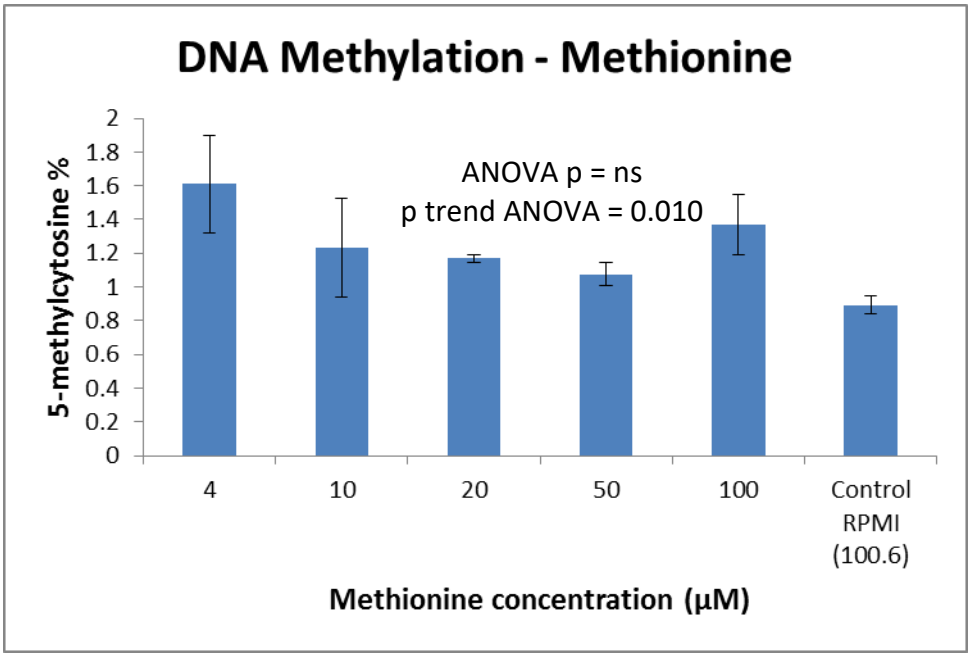


Figure 5.5: Effect of methionine concentrations on DNA methylation in HT29 cells cultured for 14 days. Data expressed as mean ± SE of triplicates.

Table 5.4: Post Hoc comparison using Multiple Comparison Bonferroni Test to determine significance of difference between methionine concentrations on DNA methylation in HT29 cells. Data shown as p value. NA = not applicable.

Methionine concentration (μM)	**4**	**10**	**20**	**50**	**100**	**Control RPMI (100.6)**
4	NA	1.000	1.000	1.000	0.188	0.329
10		NA	1.000	1.000	1.000	1.000
20			NA	1.000	1.000	1.000
50				NA	1.000	1.000
100					NA	1.000
Control RPMI (100.6)						NA

5.5.3 Correlation

Pearson correlation analysis indicated that there was a significant positive relationship between telomere length and DNA methylation status in the methionine experiment (p value = 0.037, Pearson correlation = 0.509), when data from all concentrations combined. There was a non-significant positive relationship (p value = 0.410, Pearson correlation = 0.214) between telomere length and DNA methylation status in cells from the folic acid experiment, when data from all concentrations combined. A non-significant positive relationship (p value = 0.061, Pearson correlation = 0.340) between telomere length and DNA methylation status was observed, when data from all experiments (both folic acid and methionine) are combined.

5.6 Discussion

Dietary components, such as folate and methionine may play important roles in the epigenetic mechanisms such as DNA methylation which are involved in their ability to impact gene expression [209]. Data from the present study indicate that folate and methionine depletion may increase telomere length and DNA methylation in HT29 cells *in vitro*. Furthermore, telomere length and DNA methylation were positively correlated with each other. These results do not agree with hypothesis number 3 that folic acid or methionine deficiency cause global DNA hypomethylation. Nevertheless, the results support hypothesis number 4 that lower folic acid or methionine concentration is associated with longer telomeres in the short term 14 day period.

Similar observations of folic acid depletion being associated with increasing telomere length was reported in a study by Bull et al. (2014) [199]. It was found that telomere length was significantly increased in WIL2-NS cells cultured in folic acid depleted medium (with concentration of 30 nM of folic acid) for 14 days, compared to control RPMI [199]. It is plausible that the increase in telomere length observed with the depletion of folate and methionine in the present study, may be due to a critical decrease in heterochromatic histone methylation and subtelomeric DNA methylation, leading to open chromatin [218, 219]. The structure of an open chromatin allows greater access by telomerase and other proteins involved in telomere elongation [220].

It was proposed that folate status plays a role in telomere length by affecting DNA integrity and epigenetic regulation of telomere length through DNA methylation ([212, 214, 221] and as reviewed in [222]). Folate and methionine are directly involved in DNA methylation via one-carbon metabolism. In the metabolism pathway, methionine is essential as a methyl group donor for DNA methylation process in cells. Thus theoretically, excess methionine might therefore be predicted to facilitate DNA methylation [223]. Methionine is converted to SAM in the pathway, and then SAM donates its labile methyl groups to methylation reactions. Methionine is also regenerated from homocysteine with the presence of 5-methyltetrahydrofolate. In the condition of limited folate supply, however, reduced availability of methyltetrahydrofolate decreases the biosynthesis of SAM. Consequently, this condition limits the availability of methyl groups for methylation process [224]. This was supported by previous studies showing decreased DNA methylation

associated with folate deficiency in humans [129, 225], animal model [226] and in cell lines [227]. The inhibition of DNA methylation associated with folate depletion has also been associated with increased cancer susceptibility [209]. Thus, in this study, it was hypothesised that folate or methionine deficiency decreases DNA methylation in HT29 cell lines. However, results obtained did not support the hypothesis.

Results from this study show that folate and methionine depletion tended to increase DNA methylation after 14 days of culture (non-significant data). The trend was particularly evident in HT29 treated with methionine deficiency. It is suggested that excess of methionine may actually impair DNA methylation, due to the inhibition of remethylation of homocysteine [223]. This could be because an increase in SAM inhibits MTHFR needed to make 5-MeTHF. SAM is a known inhibitor of MTHFR necessary to convert folic acid to 5-MeTHF. Furthermore, when SAM increases, so does SAH and SAH is a powerful inhibitor of DNA methyl transferase. Available data from previous studies suggested that the effect of folate and methionine deficiency on DNA methylation is site and gene-specific. In a study by Jhaveri et al. (2001), for example, wild-type human nasopharyngeal epidermoid carcinoma KB cells cultured in folate deficient medium (2-10 nM folic acid) was associated with paradoxical hypermethylation in a 5′ CpG island (by 40%), and resulting in down-regulation of the H-cadherin gene compared with cells cultured in folate replete medium (2000 nM folic acid) [228]. Meanwhile, study by Bull et al. (2014) reported that folic acid depletion (at 30 nM) reduced global DNA methylation (expressed as 5-methylcytosine percentage) in WIL2-NS cells cultured for 14 days and 42 days, compared to control in RPMI

[199]. Several studies on *in vivo* supplementation with folic acid in humans failed to observe an increase in global DNA methylation [225, 229]. In a population-based intervention study among women of reproductive age by Crider et al. (2011), no global DNA methylation changes were associated with up to 4000 μg/ day folic acid supplementation for six months [229]. An earlier study by Rampersaud et al. (2000) was conducted in healthy postmenopausal women who consumed a moderately folate depleted diet (118 μg/day folate) for 7 weeks, followed by 7 weeks of folate repletion with 200 or 415 μg/day. No changes in global DNA methylation were detected in either group over the 7 week folate repletion period, suggesting that normalization of DNA methylation after moderate folate depletion may be delayed [225].

Bull et al. (2014) proposed the possible pathway by which folate deficiency could lead to increase in telomere length is through the effects of hypomethylation, potentially altering chromatin at telomeres, allowing telomerase access to its substrate, thus increasing telomere length [199]. In this study with HT29 cells neither folic acid nor methionine deficiency resulted in global DNA hypomethylation, however, significant increases in telomere length were observed. These data suggest the possibility of an alternative mechanism in this cell type. Positive correlation was found between telomere length and DNA methylation in the present study, which differs to previous investigations that show a negative relationship between DNA methylation and telomere length [197-199, 202]. Further contradictory data was shown by Lee (2009) suggested that there is no

significant direct correlation between subtelomeric DNA methylation and telomere length in human cancer cell lines, hepatoma Hep3B cells [201].

The relationship of telomere length with folate and methionine is not linear because of evidence on the one hand suggesting that DNA hypomethylation may be associated with telomere lengthening and on the other hand folate deficiency induced terminal deletion results in removal of telomeres [199]. Furthermore telomere length decreases with each nuclear division due to the end-replication problem [230] which may explain why in Figure 5.2 and 5.3 telomere length got shorter at the highest folate and methionine concentration with highest proliferation rate (i.e. in 2655 nM folic acid and 100.6 µM methionine in control RPMI1640).

In conclusion, these *in vitro* studies demonstrate that (i) folate and methionine depletion may increase telomere length and global DNA methylation in HT29 cells and (ii) telomere length is positively correlated with DNA methylation. These findings support the hypothesis that folic acid or methionine deficiencies are associated with increased telomere length in HT29 cell lines. However the hyphothesis of folic acid or methionine deficiency decreasing DNA methylation in HT29 cell lines was not supported.

Chapter 6:

In Vivo Studies – I

The Effect of Folate and Methionine on Cancer Development in the $Apc^{Min/+}$ Mouse Model

6.1 Objective

The aim of this study was to assess the effect of supplementing a western-style diet with dietary folic acid and/or methionine on intestinal tumour development in the $Apc^{Min/+}$ mouse.

6.2 Hypotheses

1. Excess dietary folic acid and/or methionine decreases cancer incidence and growth in the small intestine and colon of the $Apc^{Min/+}$ model.

6.3 Introduction

The impact of dietary practice on cancer incidence has been addressed by researchers for many years. A "Western-style" dietary pattern in humans, defined as one with high fat (approximately 40 % of total calories), inadequate (less than recommended intake) calcium, vitamin D and methyl donor nutrients (folic acid, methionine and choline), has been proposed as a risk factor for developing intestinal tumours [136, 231]. In rodent studies, Western-style diet (WD) containing low calcium, vitamin D and high fat was found to result in hyperproliferation, hyperplasia [231] and whole crypt dysplasia development

[232] in the colon, but not colonic adenoma or adenocarcinoma. WD was then further modified to the New Western-style Diet (NWD) with decreased levels of nutrients required for biochemical reactions involving methyl group deficiency (i.e. folic acid, methionine, choline and vitamin B12) [136]. NWD administration resulted in adenoma and carcinoma development in the colon [136].

The influence of folate and methionine on intestinal tumour development in the $Apc^{Min/+}$ mouse model and the relevance of using this mouse model have been discussed in detail in Chapter 2 [93]. *In vitro* investigation on the effects of various folate and methionine concentrations on cancer cell growth (in HT29 cells) has been carried out and discussed in Chapter 4. Restriction of folate or methionine was observed to prevent excessive proliferation in HT29 cells. However, *in vitro* models do not replicate the physiological conditions of the *in vivo* intestinal cell environment and as a consequence, this part of study was carried out to investigate the *in vivo* impact of dietary folate and methionine on the growth of intestinal tumours in the $Apc^{Min/+}$ mouse model. This study was done to determine if supplementation of a western diet with folic acid and methionine, at intakes above the minimum requirement for growth, can prevent the growth of tumours of the gastrointestinal tract, and the associated physiological changes. Apart from investigating the effects of folate or methionine supplementation, this study also examined at the effects of folate and methionine supplementation in combination. Most of the previous studies reviewed in Chapter 2 (Table 2.1) [93] only assessed the effects of folate or methionine independently,

but as they both act through the same biochemical pathway it is important to study them in combination.

The $Apc^{Min/+}$ mouse model has been used extensively to study the dietary effects on intestinal cancer. Despite this, the limitation to this model is that the majority of tumours develop in the small intestine, with fewer in the colon. The mice mainly develop small intestinal polyps in contrast to humans for whom intestinal cancer develops mainly in the colon [94]. Because of this limitation, in our study the mice were fed with 'New Western Diet' (NWD) which has been shown to increase the number of colonic tumours in mice intestinal tumours [136, 231, 233] which makes this model more relevant to the human condition. A study by Fan et al. (2007) reported that after 9 weeks supplementation of NWD to the $Apc^{Min/+}$ mice, tumours were increased primarily in the colon, with tumour multiplicity increasing by 5.3 fold and tumour volume increasing by 6.7 fold [233].

6.4 Material and Methods

6.4.1 Mouse Model

All mice in this study were supplied internally from "Breeding $APC^{Min/+}$ mice" program (ethics no: 721-12/09, Dr. Erin Symonds). The breeding program involved mating male $APC^{Min/+}$ (Jackson Laboratories, Bar Harbor, ME) with female C57BL/6 mice (ARC, Perth, Australia). From this breeding program, approximately 50% of the surviving pups were heterozygotes for the dominant Min gene mutation for intestinal cancer ($APC^{Min/+}$) and the other 50% were healthy wild type (WT) homozygotes, of mixed sex. All have a C57BL/6

background. C57BL/6 mice are extensively used as a general strain and background strain for the generation of congenics with both induced and spontaneous mutations. All mice prior to receipt were genotyped.

6.4.2 Genotyping

Mice genotyping was carried out as has been reported by Symonds and Fenech (2012) [234]. DNA was isolated from fresh fecal samples. For each DNA sample, real-time PCR was set up containing three primers (specific primer for WT, specific primer for APC^{Min}, and a common antisense primer) to detect each allele. Real time PCR was done in a final volume of 20 µL/sample, using 10 µL SYBR green master mix (Applied Biosystems, Victoria, Australia), 0.25 µL WT primer, 0.25 µL APC^{Min} primer, 0.5 µL common antisense, and 40 ng DNA. A negative PCR control contained all reagents except DNA. Cycling conditions were: 10 min at 95°C, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. The dissociation stage was performed by 95° C for 15 seconds, 55°C for 30 seconds, and 95°C for 15 seconds. PCR products were run on 2% (w/v) agarose gel in 0.5 × TAE buffer (Tris, acetic acid, ethylenediaminetetraacetic acid) containing gel red (Jomar Diagnostics, Stepney, South Australia). WT mice had a single band (619 bp), while $APC^{Min/+}$ mice had two bands.

6.4.3 Study Design

Approval for this study was obtained from CSIRO (ethics number 735-01/10) and University of Adelaide (M-069-2008) animal research ethics committees. Comparative experiments were performed between $APC^{Min/+}$ mice and age-matched and gender-matched WT littermates as healthy control mice as summarized in Figure 6.1. The effect of increased dietary folic acid and/or methionine intake in the $Apc^{Min/+}$ model to protect against tumour development when administered at age of 3 weeks at weaning until age of 13 weeks was assessed, with WT mice used as controls. Each treatment regime group consisted of a control group (WT mice) and a cancer risk group ($APC^{Min/+}$ mice) of mixed sex. WT mice were used to assess the effect of increased dietary folic acid and methionine on the physiology of normal mice. The rationale of using New Western Diet (NWD), a modified version of AIN-93G diet for rodents was to test the effect of low or high folate and methionine in the context of other risk factors in the Western diet (i.e. high fat, low calcium, low methyl donor nutrients (folic acid, methionine and choline) and low vitamin D3).

A total number of 113 mice were used in this study; comprising 55 $APC^{Min/+}$ mice and 58 WT mice. All mice were randomised to receive one of the four diet treatments; New Western Diet (NWD) as control diet, New Western Diet with additional folic acid (NWD+FA), New Western Diet with additional methionine (NWD+M), and New Western Diet with additional folate and methionine (NWD+FA+M). Average sample size of each group was 14.13 ± 0.83 (mean ± standard deviation (SD)). Based on Moser (1990), total average tumour number per mouse is expected to be 29 ± 10 (mean ± SD) [85]. The sample size of 14 mice

per group gives a statistical power of 82% for a one tailed test at the 5% significance level to detect a 30% decrease in the number of tumours. The mice on the supplemented diets were compared to groups that received control diet and all mice were sacrificed when aged 13 weeks (average of 94 ± 1 days, mean ± SD). Figure 6.1 explains the study design.

This study was carried out using a 2X2 factorial design aimed at investigating the independent and combined effect of low or high dietary folic acid and/or methionine as shown in Table 6.1. NWD contained 0.23 mg of folic acid/kg diet and 4.6 g of methionine/kg diet, NWD+FA contained 8 mg of folic acid/kg diet and 4.6 g of methionine/kg diet, NWD+M contained 0.23 mg of folic acid/kg diet and 15 g of methionine/kg diet, whereas NWD+FA+M contained 8 mg of folic acid/kg diet and 15 g of methionine/kg diet.

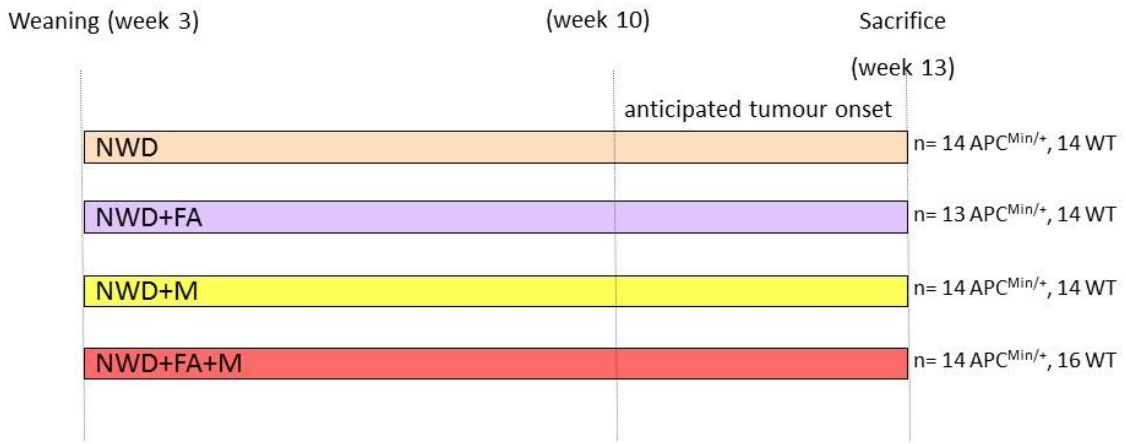


Figure 6.1: Dietary groups, study points and number of mice per group. At 3 weeks of age mice were weaned into experimental diets. At around 10 weeks of age, the onset of cancer and associated anaemia may potentially cause distress. Mice were monitored for clinical symptoms to minimise distress. At week 13, mice were sacrificed.

Table 6.1: 2X2 factorial design of study.

		Folic Acid	
		0.23 mg/kg diet	**8 mg/kg diet**
Methionine	**4.6 g/kg diet**	0.23 mg of folic acid/kg diet + 4.6 g of methionine/kg diet	8 mg of folic acid /kg diet + 4.6 g of methionine/kg diet
	15 g/kg diet	0.23 mg of folic acid/kg diet + 15 g of methionine/kg diet	8 mg of folic acid/kg diet + 15 g of methionine/kg diet

Diet containing 8 mg folic acid/kg represents folate supplement four times the basal dietary requirement (at 2mg/kg), and has consistently provided a degree of chemoprevention against colorectal cancer in previous rat studies [235, 236]. Diet containing 15 g of methionine/kg diet (equal to 1.5%) was chosen because previous study indicates that higher levels of methionine (1.8% to 3.0%) tended to cause growth repression in rats (measured by body weight gain) [237].

6.4.4 Diet

All diets pellets were prepared in the CSIRO diet facility and stored frozen at -20ºC. This study used New Western Diet (NWD) as the control diet, a modified version of AIN-93G diet for rodents which mimics risk factors in a human western population (i.e. high fat, low calcium, low methyl donor nutrients (folic acid, methionine and choline [233]) and low vitamin D_3) [231, 233].

Details of the New Western Diet (NWD) used in this study (composition shown in Table 6.2):

- 0.05% calcium was used. A study by Wargovich et al. (1990) used calcium at 0.04% (reflecting human daily intake of approximately 200-250 mg/day) of the diet in a carcinogenesis study in rats and showed a protective outcome of dietary calcium on high dietary fat promotion of chemically induced colon cancer [238].
- The 0. 11 lU/g level of vitamin D is equivalent to 220-300 IU of cholecalciferol on a diet of 2000-2700 kcal, which is lower than the US recommended dietary allowance (600 IU per day) [239].

- The protein content of NWD (20% by weight) represents approximately 18% of calories, similar to the average Western diet [231].
- Total dietary fibre of adults (US) is approximately 12 g/2000 kcal [240]. In the NWD, fibre content was reduced to 2%, which is equivalent to approximately 9 g/day (on a 2000 kcal basic diet), to mimic the relative human dietary fibre intake.
- Adult human dietary requirement of choline is approximately 0.25 mg/kcal diet [241]. The level used in the NWD formulation provided approximately 0.26 mg choline/kcal [136].
- A fat content of 20% of the diet has been used in numerous long-term rodent studies, mainly with chemical carcinogens to study the tumour promoting effects of high fat diet. 20% fat content represents a level of 40% of total calories derived from fat, which is consistent with a human western-style diet [231].

Table 6.2: Composition of New Western Diet with/without Added Folic Acid and/or Methionine.

	NWD* g/kg diet	**NWD+FA g/kg diet**	**NWD+M g/kg diet**	**NWD+FA+M g/kg diet**
Cornstarch	399.5	399.5	399.5	399.5
Casein (>85% protein)	200	200	200	200
(Methionine)**	(4.6)	(4.6)	(4.6)	(4.6)
L-cystine	3	3	3	3
Sucrose	131.3	131.3	131.3	131.3
Lard	100	100	100	100
Sunflower oil (no additives)	100	100	100	100
Fibre (alpha cellulose)	20	20	20	20
Mineral mix	35	35	35	35
Ca	0.5	0.5	0.5	0.5
Phosphorus	2.7	2.7	2.7	2.7
Vitamin mix	10	10	10	10
Folic acid***	0.00023	0.00023	0.00023	0.00023
Vitamin D_3 IU	110	110	110	110
Choline bitartrate (41.1% choline)	1.2	1.2	1.2	1.2
Additional methionine	0	0	10.4	10.4
Additional folic acid	0	0.00777	0	0.00777
Total	1000	1000	1000	1000

* Based on the NWD diet described by Newmark et al. (2001) [136].

** Estimated methionine content in casein.

*** Estimated folic acid content in vitamin mix.

6.4.5 Animal Welfare

All mice were housed within the CSIRO animal holding facility (isolation room, Animal Housing Facility). Mice were caged based on treatment, with males and females caged separately (approximately 5 per cage). Each cage contained paper bedding material and PVC tubes to enrich the environment. All mice were monitored daily in the mornings for clinical status (as described in Table 6.3) and their cages were checked in the afternoons to ensure that food and water supply was adequate and no water leakage in the cage. Noise and traffic were kept to a minimum in the room.

The onset of cancer and associated anaemia around 10 weeks of age may potentially cause distress [242, 243]. The $APC^{Min/+}$ mice were monitored daily for the clinical symptoms that are described in Table 6.3 to minimise distress. Based on clinical signs of severity scoring, no mice required euthanasia before the end of the study. Euthanasia was performed only when any mouse displayed two or more symptoms at grade 2 or above, or one or more of grade 3 or above as described in Table 6.3. At the end of study, after sacrifice and tissue collection, the carcasses were frozen prior to biohazard bin collection and incineration.

Table 6.3: Clinical signs severity score (primarily for the $APC^{Min/+}$ mice)

Signs	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Chronic weight loss compared with age-matched controls	Normal weight	1-8%	9-16%	17-25%	>25%
Acute weight loss compared to original weight	No change	1-4%	5-7%	8-10%	>11%
Degree of anaemia	Normal	Slightly pale eyes, ears, feet	Obvious pale extremities	Very and cool extremities, blood in stool	Very pale,blood in stools, depressed behaviour
Rectal bleeding	Negative	Slight	moderate	obvious	gross
Behaviour/posture/activity	Normal	Slightly hunched, sometimes on own; transiently dull	Sometimes hunched; quiet and often on own; dull	Often hunched or curled posture; isolated, does not interact with others; significantly dull	Always hunched; continually isolated from others; does not move with others when disturbed
Appearance of coat	Normal	Rough coat	Rough coat	unkempt; wounds, hair thinning	bleeding or infected wounds, or severe hair loss or self mutilation
State of hydration	Normal	5% skin inelastic to touch	7% skin remains in “pulled” position; eyes slightly depressed	10% dry mouth and more severe eye changes	>12% muscle twitching and shock
Vocalisation	Normal	Squeals when palpated	Squeals when handled	Struggles and squeals loudly when handle/palpated	Abnormal vocalisation when not disturbed
Stool consistency	firm	Mild diarrhoea	Moderate diarrhoea	Severe diarrhoea	severe diarrhoea or no material passed

6.4.6 Body Weight

All mice were monitored and had their body weight measured and recorded on Mondays, Wednesdays and Fridays from weaning until end of study. The percentage of body weight increase was calculated.

6.4.7 Food Intake Observation

Average food intake was measured on a fortnight basis. It was measured by giving a known quantity of food to the mice, and then weighing the remaining food 24 hours later. Food intake was averaged for the cage of mice. This indicated if consumption is altered by the addition of folate and methionine in the pellets.

6.4.8 Sacrifice of Mice at End of Experiments

At age 13 weeks, all mice were sacrificed. This was performed via anaesthesia with isoflurane (Veterinary Companies of Australia Pty Ltd, NSW, Australia), followed by CO_2 asphyxiation and blood collection via cardiac puncture and posterior vena cava. To ensure that a mouse is dead, cervical dislocation was performed. The blood from the cardiac puncture and posterior vena cava were collected into a heparinised blood collection microtainer tube (BD, Australia) to prevent blood coagulation.

6.4.9 Organs Weight and Size

Liver, spleen, kidneys, small intestine, colon, stomach and caecum were collected from all mice. Weight of organs were measured and recorded. Small intestine, colon, stomach and caecum were emptied before weighing. The length of small intestine and colon were also recorded.

6.4.10 Haematocrit Measurement

Haematocrit was measured as an indicator of anaemia [243]. Blood was collected (as described in 6.4.8) and drawn into a heparinised haematocrit capillary tube and sealed with putty in one end. The capillary tube was then placed in micro-haematocrit centrifuge (Hawksley and Sons Ltd., UK) with the sealed end facing out, and spun for 5 minutes. The haematocrit was measured using a micro-haematocrit reader (Hawksley and Sons Ltd., UK) as percentage of packed cell volume.

6.4.11 Plasma Methionine and Folate Measurement

Approximately 500µL of blood collected (as described in 6.4.7) was centrifuged at 2000 g for 5 minutes to separate plasma and blood cells. The clear supernatant produced following centrifugation is designated plasma. Plasma collected was then transferred into another tube for measurement of plasma methionine and folate levels. Plasma of the same group (all male and female mice from a diet group) were pooled (with consistent volume from each mice) because not all mice yielded sufficient blood to enable individual

quantification. Samples were sent to South Australia Pathology, Adelaide, South Australia for folate and methionine quantification.

6.4.12 Tumour Measurement in $APC^{Min/+}$ Mice

i. Small Intestinal Tumour Measurement

The small intestine collected was kept moist in phosphate buffered saline (PBS). The small intestine was then divided into the proximal and distal section (divided by cutting into two segments of equal length). Each segment was opened longitudinally, further rinsed with cold PBS, and flattened with mucosal surface uppermost. The number of tumours and location in the small intestine were recorded. Peyer's patches were not included in the quantification. However, Peyer's patches were identified based on these criteria: they occur in the submucosa layer, the surface is not covered by villi, and can be removed through mucosal scraping. Only confirmed tumours were recorded. In addition, the size (in two dimension, length and width) of each tumour was measured and recorded. Tumour size were used to calculate tumour load [244]. Figure 6.2 shows small intestinal tumours in an $APC^{Min/+}$ mouse.

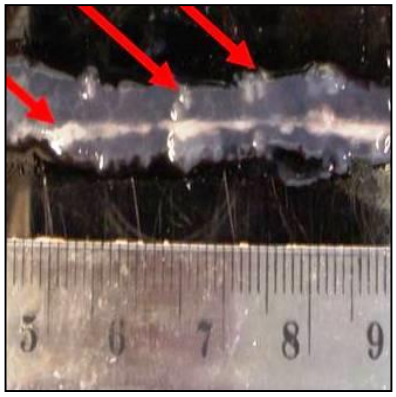


Figure 6.2: Tumours observed in the small intestine of an $APC^{Min/+}$ mouse.

ii. Colon Tumour Measurement

The dissected colon was kept moist in PBS. The colon segment was opened lengthwise, further rinsed with cold PBS, and flattened with mucosal surface uppermost. The number of tumours and location in the colon were recorded. The size (in two dimension, length and width) of each tumour was measured and recorded. Tumour load was calculated using the size measurements [244]. Figure 6.3 shows tumour in the colon in an $APC^{Min/+}$ mouse.

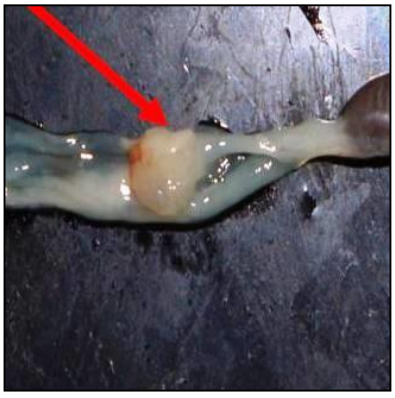


Figure 6.3: Tumour observed in the colon of an $APC^{Min/+}$ mouse.

6.4.13 Folate and Methionine Measurement in Mucosal Tissue of Small inestine

A tissue section approximately 1 cm in length was removed from the small intestine, washed in PBS and allowed to stand in PBS for 60 minutes. Tissues of the same treatment group (combined male and female mice) were pooled in this experiment. Tissue sections were then drawn in and out a syringe several times to slough off the mucosal layer. The muscle layer was discarded, and the remaining cell solution was centrifuged at 120 g for 5 minutes. Following removal of the supernatant, 100 µL of human plasma and 100 µL 0.4% ascorbic acid were added. The resulting cell suspensions were then sonicated. Samples were centrifuged at 120 g for 5 minutes and the supernatant obtained was then incubated at 37°C for 30 minutes to allow deconjugation of polyglutamated folate by conjugase in plasma. A further 200 µL of 0.4% ascorbic acid was added and samples were sent to South Australia Pathology, Adelaide, South Australia for folate and methionine quantification. The values of folate and methionine in the plasma without small intestine mucosal cells, was subtracted from the values for plasma with small intestine mucosa cells to obtain the actual level in the ephithelial cells. The results were then corrected for total protein content of the mucosal cell pellet obtained after sonication and centrifugation.

Protein was measured using the Lowry assay [245]. Pellets obtained from the centrifugation were added with 1 mL of complex-forming reagent (2% sodium carbonate, 1% copper (II) sulfate pentahydrate and 2% potassium sodium tartrate tetrahydrate in the ratio of 100:1:1). The mixture was allowed to stand at room temperature for 10 minutes. A 100 µL Folin reagent (1:2 in H_2O) was added and the solution was then votexed. The

mixture was allowed to stand for 40 minutes at room temperature before the OD was read at 595 nm. Protein concentration was determined based on a standard curve of absorbace plotted using bovine serum albumin as standard.

6.4.14 Statistical Analysis

All data were analysed with IBM SPSS Statistic 21. One Way ANOVA followed by Post Hoc Multiple Comparison Bonferroni test were used to determine the significant differences between groups for all measurement. The measurements were carried out across groups rather than by distinct treatments to provide confidence in the analyses. False Discovery Rate (FDR) is shown for multiple comparison analysis [246]. Bivariate Pearson Correlation test was carried out to determine the relationship between plasma methionine and haematocrit percentage. Two-way ANOVA analysis was carried out to determine the effects of folic acid and methionine on haematocrit level, plasma methionine, folate and methionine concentration in mucosal tissue of the small intestine (MTSI), tumour incidence and tumour load. A p value less than 0.05 was considered significant.

6.5 Results

6.5.1 Body Weight

Table 6.4 presents the average initial and final body weight recorded and also percentage of body weight increase for WT and $Apc^{Min/+}$ in all diet groups. There was no significant difference of body weight observed between all diet groups in WT (p value = 1.000), or in $Apc^{Min/+}$ mice (p value = 0.773-1.000). Mean mouse weights were in the range of 7.76 – 9.37 g at the beginning of study, and ranged from 22.51 – 25.78 g at the end of study. Average percentages of weight increased recorded were 168.17 % - 214.92 %. Average (± SD) final weight recorded for all WT mice was 24.85 ± .14 g and 23.17 ± 3.43 g for $Apc^{Min/+}$ mice. There was no significant difference between the average final weight of WT and $Apc^{Min/+}$ mice (T-test p value = 0.093).

6.5.2 Food Intake

Figure 6.4 shows the average food intake of mice observed in 24 hours. There was no significant difference in 24-hour food intake between all diet groups in WT (multiple comparison p value = 0.220-1.000) and in $Apc^{Min/+}$ mice (p value = 1.000). Average (± SD) 24-hour food intake of WT mice was 2.78 ± 0.57 g and 2.53 ± 0.42 g (mean ± SD) for $Apc^{Min/+}$ mice (T-test p value = 0.271).

Table 6.4: Mean body weight and percentage of body weight increase in WT and $Apc^{Min/+}$ mice, before and after 10 weeks on treatment diets.

Genotype	Diet	Initial Body Weight (g)	Final Body Weight (g)	% Increased*
WT	NWD	9.37 ± 2.12	25.78 ± 5.25	184.20 ± 63.48
	NWD+FA	8.81 ± 1.77	25.07 ± 4.44	189.45 ± 45.41
	NWD+M	8.20 ± 0.80	23.96 ± 2.74	193.93 ± 36.51
	NWD+FA+M	8.90 ± 2.03	24.62 ± 3.98	184.06 ± 47.59
$Apc^{Min/+}$	NWD	9.09 ± 3.16	23.03 ± 5.07	172.53 ± 89.11
	NWD+FA	7.76 ± 1.45	23.13 ± 2.05	214.92 ± 100.78
	NWD+M	8.61 ± 2.04	22.51 ± 2.34	172.07 ± 54.38
	NWD+FA+M	9.31 ± 2.25	24.01 ± 3.55	168.17 ± 63.05

Data expressed as mean ± SD.

*ANOVA p value = ns for comparison between the four dietary groups for each genotype.

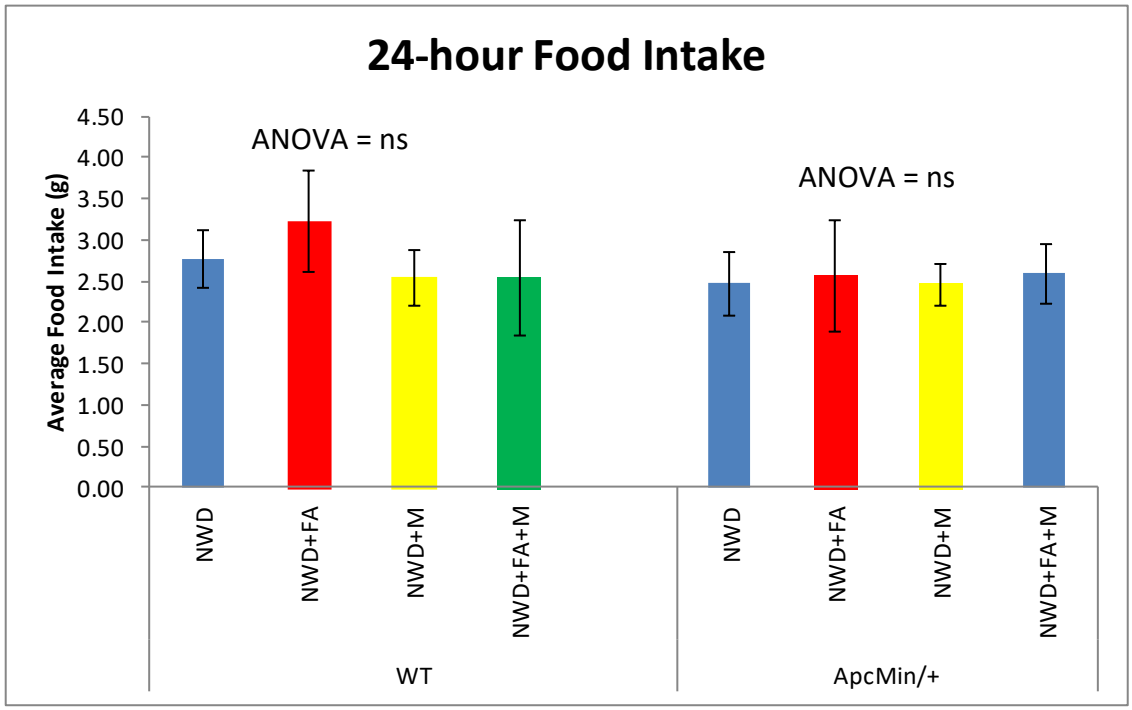


Figure 6.4: Average 24-hour food intake in WT and $Apc^{Min/+}$ mice. Data expressed as mean ± SD.

6.5.3 Organ Weight and Size

Weight of organs including liver, spleen, kidneys, small intestine, colon, stomach and caecum, and length of small intestine and colon were recorded and are represented in Table 6.5. In WT groups, no significant difference was observed for liver weight (p value= 1.00), kidneys weight (multiple comparison p value = 0.525-1.000), small intestine weight (p value = 0.693 – 1.000), stomach weight (p value = 0.475 – 1.000), or colon length (p value = 0.203 – 1.000). There was significant difference in spleen weight between WT mice fed with NWD+FA and NWD+M at p value = 0.027. The length of small intestine in WT mice fed with NWD+FA was lower than in the group fed with NWD, at p value = 0.048. For weight of colon, there was a significant lower weight recorded for group fed with NWD+FA compared to NWD group (p value = 0.021), however no significant differences were recorded between other treatment groups (p value = 0.401 – 1.000). For caecum weight, there was significant lower weight recorded for group fed with NWD+FA compared to NWD (p value = 0.000) and NWD+FA+M (p value = 0.010) groups.

In $Apc^{Min/+}$ groups, there was no significant difference observed for liver weight (p value = 1.000), kidneys weight (p value = 0.507 – 1.000), small intestine weight (p value = 0.741 – 1.000), and stomach weight (p value = 0.555-1.000). For spleen weight, there was a significant lower weight observed in NWD+FA group compare to NWD group (p value = 0.047). However, there was no significant difference found between NWD group and groups supplemented with NWD+M and NWD+FA+M (p value = 0.675 – 1.000). The length of small intestine in NWD+FA group was lower compared to NWD+M (p value = 0.000) and

NWD+FA+M (p value = 0.028) groups. For colon length, there was a significant difference observed between NWD and NWD+M groups at p value = 0.010, as well as between NWD+FA and groups fed with NWD+M (p value = 0.000) and NWD+FA+M (p=0.048). In terms of colon weight, the group fed with NWD+FA had lower weight compared to groups fed with NWD (p value = 0.026), NWD+M (p value = 0.020) and NWD+FA+M (p value = 0.009). A similar observation was found in caecum weight with the group fed with NWD+FA having marked lower weight compared to groups fed with NWD (p value = 0.000), NWD+M (p value = 0.002) and NWD+FA+M (p value = 0.000).

Table 6.5: Mean weight (expressed in g) and size (expressed in cm for length) of organs in WT and $Apc^{Min/+}$ mice.

	NWD	NWD+FA	NWD+M	NWD+FA+M
WT				
Liver Weight	1.45 ± 0.23	1.42 ± 0.34	1.42 ± 0.24	1.34 ± 0.24
Spleen Weight	0.10 ± 0.01 [a,b]	0.11 ± 0.03 [a]	0.09 ± 0.01 [b]	0.11 ± 0.03 [a,b]
Kidneys Weight	0.37 ± 0.06	0.36 ± 0.09	0.34 ± 0.06	0.38 ± 0.05
Small Intestine Length	36.07 ± 2.93 [a]	33.64 ± 2.20 [b]	34.71 ± 1.93 [a,b]	34.69 ± 2.17 [a,b]
Small Intestine Weight	0.45 ± 0.08	0.46 ± 0.13	0.50 ± 0.08	0.44 ± 0.08
Colon Length	7.36 ± 0.81	6.68 ± 0.83	7.15 ± 0.94	6.84 ± 0.72
Colon Weight	0.29 ± 0.03 [a]	0.15 ± 0.03 [b]	0.18 ± 0.03 [a,b]	0.17 ± 0.03 [a,b]
Stomach Weight	0.12 ± 0.04	0.11 ± 0.01	0.10 ± 0.02	0.12 ± 0.02
Caecum Weight	0.10± 0.03 [a]	0.05 ± 0.02 [b]	0.07 ± 0.03 [a,b]	0.09 ± 0.02 [a]
$Apc^{Min/+}$				
Liver Weight	1.32 ± 0.29	1.32 ± 0.18	1.19 ± 0.49	1.43 ± 0.25
Spleen Weight	0.25 ± 0.10 [a]	0.14 ± 0.03 [b]	0.21 ± 0.14 [a,b]	0.18 ± 0.11 [a,b]
Kidneys Weight	0.33 ± 0.08	0.39 ± 0.28	0.29 ± 0.04	0.34 ± 0.09
Small Intestine Length	34.43 ± 2.43 [a,b]	32.69 ± 1.44 [a]	36.61 ± 1.98 [b]	35.14 ± 2.53 [b]
Small Intestine Weight	0.55 ± 0.08	0.50 ± 0.29	0.46 ± 0.11	0.56 ± 0.16
Colon Length	6.93 ± 0.85 [a,b,d]	6.67 ± 0.66 [a,b]	8.01 ± 0.54 [c,d]	7.43 ± 0.76 [a,c,d]
Colon Weight	0.20 ± 0.04 [a]	0.15 ± 0.03 [b]	0.21 ± 0.05 [a]	0.20 ± 0.05 [a]
Stomach Weight	0.11 ± 0.02	0.11 ± 0.02	0.10 ± 0.02	0.11 ± 0.02
Caecum Weight	0.09 ± 0.03 [a]	0.05 ± 0.02 [b]	0.08 ± 0.01 [a]	0.10 ± 0.02 [a]

Data expressed as mean ± SD.

Groups sharing the same letter within a row are not significantly different.

Groups not sharing the same letter within a row are significantly different.

6.5.4 Haematocrit Level

Figure 6.5 displays the average of haematocrit (%) observed in all WT and $Apc^{Min/+}$ groups at the end of study. In WT group, there was a significant higher haematocrit in NWD+M group compared to NWD group (p value = 0.042, FDR = 0.126). Meanwhile in $Apc^{Min/+}$ group, there was no significant difference observed between all diet groups (p value = 0.082-1.000, FDR = 0.246 – 1.000). Average haematocrit for all WT mice was 30.79 ± 12.00 %, and 25.65 ± 11.17 % for $Apc^{Min/+}$ mice. There was no significant difference between the average haematocrit (%) in WT and $Apc^{Min/+}$ mice (T-test p value = 0.407), however there was a consistent trend for lower haematocrit in $Apc^{Min/+}$ mice.

Table 6.6 shows results of Two Way ANOVA analysis examining at the interaction of folic acid and methionine supplementation on haematocrit level. Results from WT and $Apc^{Min/+}$ combined data showed a significant interaction between folic acid and methionine supplementation on increasing haematocrit level (p value = 0.004), as well as effect of methionine supplementation (p value = 0.025). Two Way ANOVA analysis on WT data showed there was a significant effect of methionine supplementation (p value = 0.041), while analysis of $Apc^{Min/+}$ data indicated significant interaction between folic acid and methionine supplementation on haematocrit level (p value = 0.025).

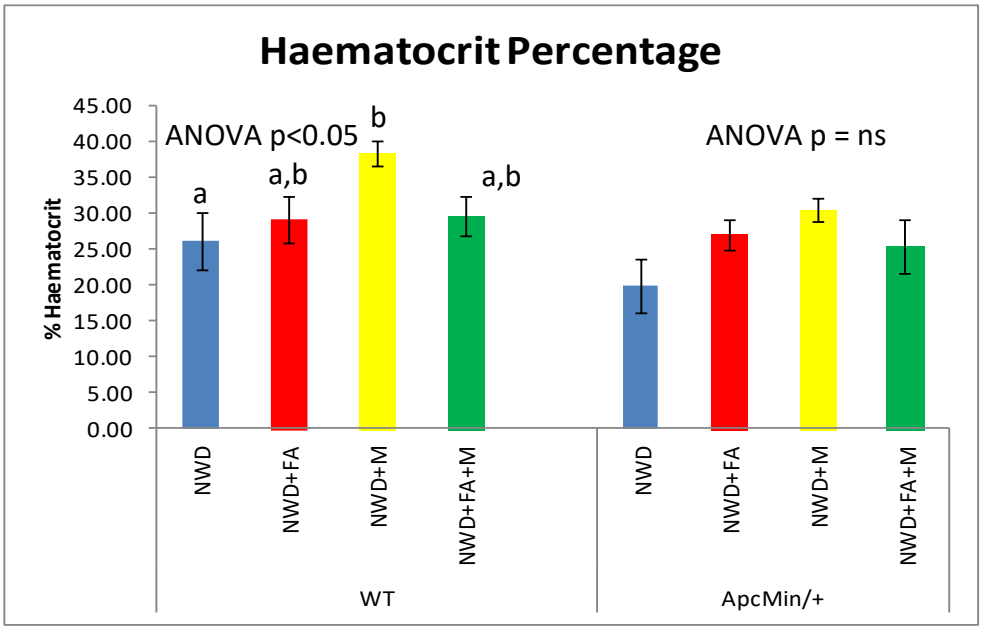


Figure 6.5: Average haematocrit percentage in WT and $Apc^{Min/+}$ mice. Data expressed as mean ± SE.

Table 6.6: Two Way ANOVA results for folic acid and methionine interaction on haematocrit percentage.

Outcome	Source of Variation	P value
Haematocrit percentage in WT and $Apc^{Min/+}$ combined	Folic acid	0.586
	Methionine	0.025*
	Folic acid and Methionine	0.004*
Haematocrit percentage in WT	Folic acid	0.363
	Methionine	0.041*
	Folic acid and Methionine	0.058
Haematocrit percentage in $Apc^{Min/+}$	Folic acid	0.962
	Methionine	0.263
	Folic acid and Methionine	0.025*

*ANOVA $p < 0.05$

6.5.5 Plasma Folate and Methionine

Figure 6.6 shows the average plasma methionine levels in WT and $Apc^{Min/+}$ mice for all diet groups. In the WT group, there were significantly higher levels observed in the groups with additional methionine supplementation. Groups fed with NWD+M and NWD+FA+M had higher plasma methionine levels compared to NWD group (p values of 0.000, 0.005 and FDR = 0.000, 0.008 respectively), as well as when compared to group fed with NWD+FA (p values of 0.00, 0.036 and FDR = 0.000, 0.054 respectively). In $Apc^{Min/+}$ groups, NWD+M showed highest level of plasma methionine compared to NWD (p value = 0.000, FDR = 0.000), NWD+FA (p value = 0.000, FDR = 0.000) and NWD+FA+M (p value = 0.001, FDR = 0.001).

Table 6.7 displays results of Two Way ANOVA analysis examining at the interaction of folic acid and methionine supplementation on plasma methionine level. Analysis on combine data from WT and $Apc^{Min/+}$ experiments showed that there was significant interaction between folic acid and methionine supplementation (p value = 0.000), as well as effect of folic acid supplementation (p value = 0.010) and methionine supplementation (p value = 0.000) on plasma methionine level. Analysis of WT mice data displayed significant effect of methionine supplementation (p value = 0.000) and significant interaction between folic acid and methionine supplementation (p value = 0.046). Meanwhile, analysis on $Apc^{Min/+}$ mice data showed significant effect of folic acid supplementation (p value = 0.006), as well as methionine supplementation (p value = 0.000) on plasma methionine level and

there was a significant interaction between folic acid and methionine supplementation (p value = 0.02) on plasma methionine level in $Apc^{Min/+}$.

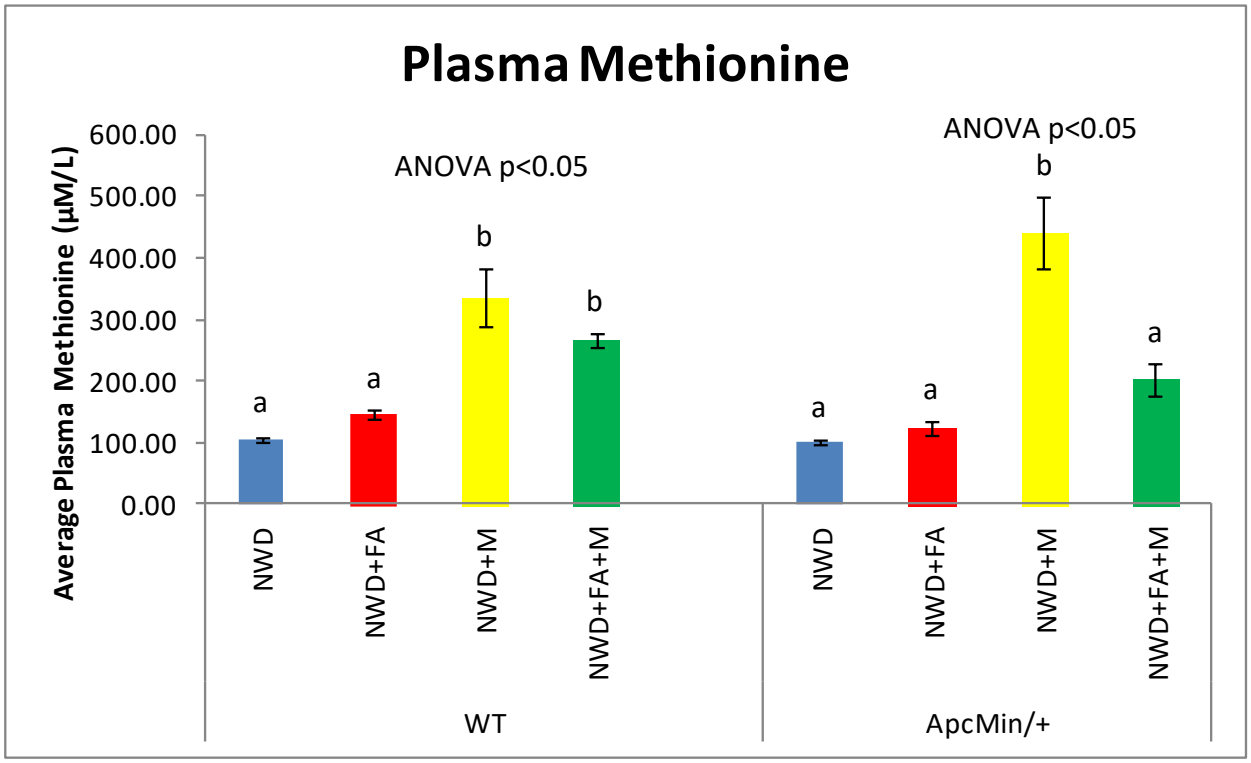


Figure 6.6: Average plasma methionine level in WT and $Apc^{Min/+}$ mice (expressed as µM/L).

Data expressed as mean ± SE.

Table 6.7: Two Way ANOVA results for folic acid and methionine interaction on plasma methionine level.

Outcome	Source of Variation	P value
Plasma methionine level in WT and $Apc^{Min/+}$ combined	Folic acid	0.010*
	Methionine	0.000*
	Folic acid and Methionine	0.000*
Plasma methionine level in WT	Folic acid	0.563
	Methionine	0.000*
	Folic acid and Methionine	0.046*
Plasma methionine level in $Apc^{Min/+}$	Folic acid	0.006*
	Methionine	0.000*
	Folic acid and Methionine	0.002*

*ANOVA $p < 0.05$

Supplementation of additional methionine in the diet resulted in significant higher plasma levels of methionine compared to control diet, observed in both genotype groups. Folate quantification in the plasma was over the measurement range available (> 45 nM/L) in South Australia pathology laboratory and unavailable to be reported. Similar result (over the measurement range available) was obtained with plasma sample being diluted, and there was insufficient plasma sample available for further dilution.

6.5.6 Folate and Methionine Concentration in Mucosal Tissue of the Small Intestine (MTSI)

Average MTSI folate levels (nM / mg protein) in WT and $Apc^{Min/+}$ mice for all diet groups are shown in Figure 6.7. In the WT group, there were significant differences observed between NWD group and groups fed with NWD+FA (p value = 0.001, FDR = 0.003) and NWD+FA+M (p value = 0.010, FDR = 0.015). Significant differences were also found between NWD+M group and groups fed with NWD+FA (p value = 0.000, FDR = 0.000) and NWD+FA+M (p value = 0.000, FDR = 0.000). In $Apc^{Min/+}$ group, there were significant differences observed between NWD+FA group and groups fed with NWD+M (p value = 0.015, FDR = 0.0315) and NWD+FA+M (p value = 0.021, FDR = 0.0315).

Average MTSI methionine levels (µg / mg protein) in WT and $Apc^{Min/+}$ mice for all diet groups are shown in Figure 6.8. There was no significant difference observed in WT group (p value = 1.000, FDR = 1.000). Whereas, in $Apc^{Min/+}$ group, there was significant lower level observed in NWD+FA+M group than NWD group (p value =0.021, FDR = 0.063). The

results suggest a different response in MTSI folate and methionine status in response to folic acid and/or methionine supplementation depending on genotype.

Table 6.8 displays results of Two Way ANOVA analysis looking at the interaction of folic acid and methionine supplementation on folate and methionine concentration in MTSI. Analysis results on combined WT and $Apc^{Min/+}$ data showed that there was a significance effect of folic acid (p value = 0.042) supplementation on folate and methionine concentration in MTSI. Analysis on WT data indicated that there were significant effects of supplementing folic acid (p value = 0.000) and methionine (p value = 0.007) on MTSI folate level. Analysis on $Apc^{Min/+}$ data showed methionine supplementation had significant effect (p value = 0.004) on MTSI folate level, whereas folic acid supplementation had significant effect (p value = 0.006) on MTSI methionine level.

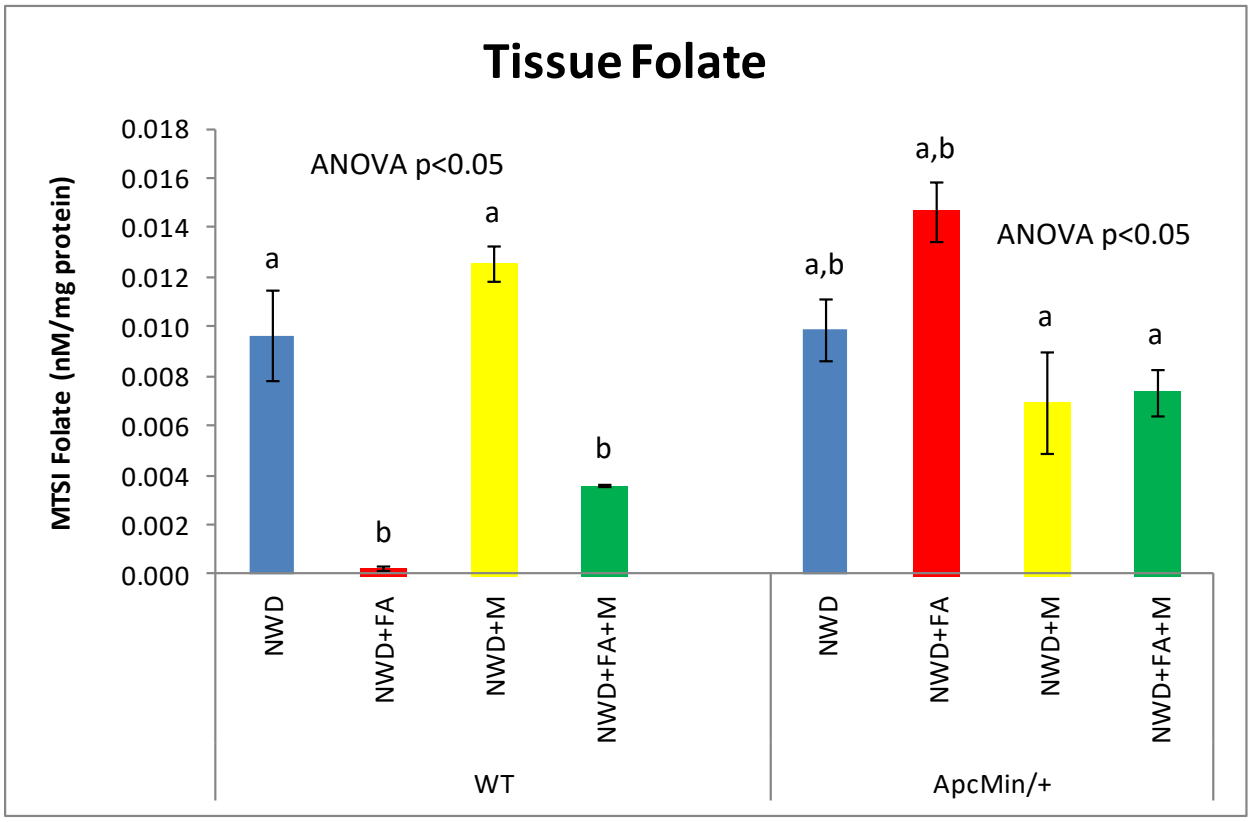


Figure 6.7: Average MTSI folate level in WT and $Apc^{Min/+}$ mice (expressed as nM/mg protein). Data expressed as mean ± SE.

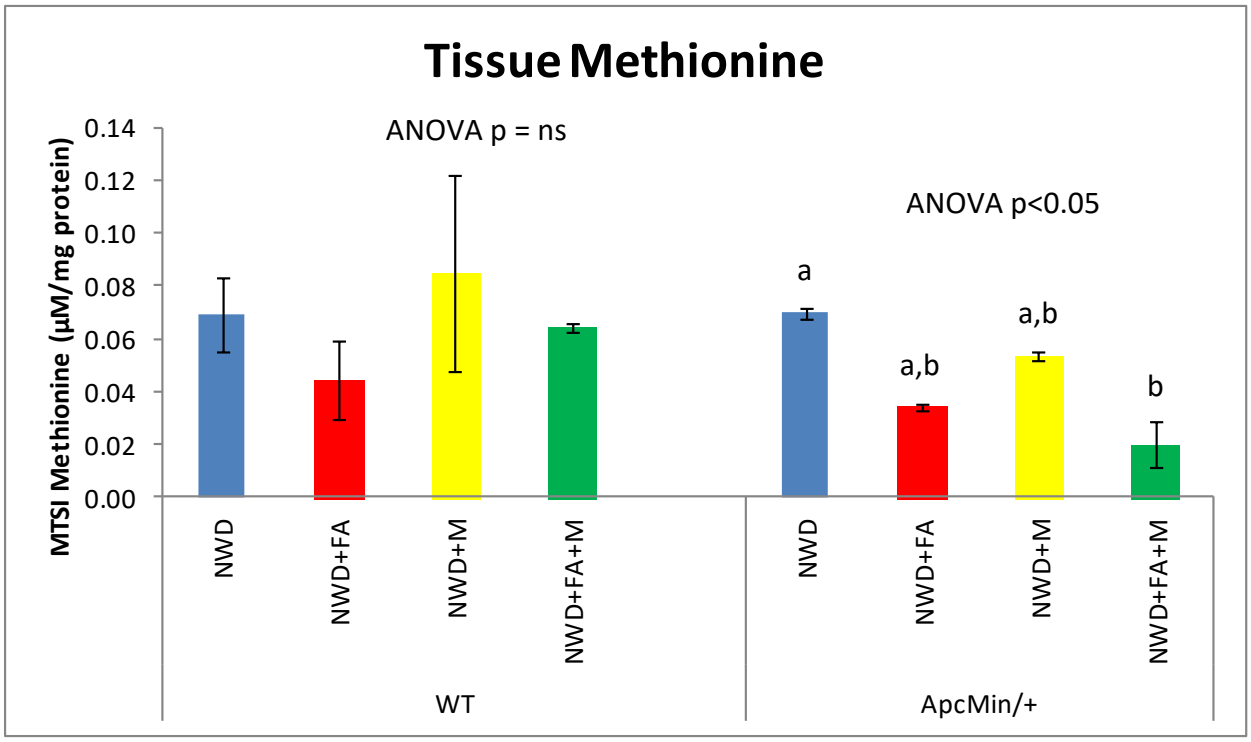


Figure 6.8: Average MTSI methionine level in WT and $Apc^{Min/+}$ mice (expressed as µM/mg protein). Data expressed as mean ± SE.

Table 6.8: Two Way ANOVA results for folic acid and methionine interaction on folate and methionine concentration in mucosal tissue of the small intestine (MTSI).

Outcome	Source of Variation	P value
MTSI folate in WT and $Apc^{Min/+}$ combined	Folic acid	0.272
	Methionine	0.253
	Folic acid and Methionine	0.258
MTSI folate in WT	Folic acid	0.000*
	Methionine	0.007*
	Folic acid and Methionine	0.813
MTSI folate in $Apc^{Min/+}$	Folic acid	0.093
	Methionine	0.004*
	Folic acid and Methionine	0.149
MTSI methionine in WT and $Apc^{Min/+}$ combined	Folic acid	0.042*
	Methionine	0.832
	Folic acid and Methionine	0.895
MTSI methionine in WT	Folic acid	0.302
	Methionine	0.421
	Folic acid and Methionine	0.917
MTSI methionine in $Apc^{Min/+}$	Folic acid	0.006*
	Methionine	0.109
	Folic acid and Methionine	0.919

*ANOVA $p < 0.05$

6.5.7 Intestinal Tumour Incidence in the $Apc^{Min/+}$ mice

Figure 6.9 displays the average tumour numbers recorded in the intestine of $Apc^{Min/+}$ mice for all diet treatment groups. Data displays as average of total tumour number in small intestine and colon, and average tumour number in each specific area; colon, small intestine, duodenum, jejunum, mid jejunum and ileum. There was no significant difference between diet groups for average total tumour recorded (multiple comparison p value = 0.132 – 1.00, FDR = 0.281 – 1.000). Similar observation was found for the average tumour number in colon (p value = 0.604 – 1.000, FDR = 1.000), small intestine (p value 0.141 – 1.00, FDR = 0.318 – 1.000), duodenum (p value = 1.00, FDR = 1.000), jejunum (p value = 0.415 – 1.00, FDR = 1.000), and mid jejunum (p value = 0.274 – 1.00, FDR = 0.822 – 1.000). Interestingly, supplementation of FA in the NWD+FA group showed significant reduction of average tumour number in ileum, compared to group fed with NWD with p value = 0.040, FDR = 0.120. However, there was no significant difference observed between NWD and NWD+M (p value = 0.130, FDR = 0.195) and NWD+FA+M (p value = 0.646, FDR = 0.646).

Despite the statistically non-significant effects by One Way ANOVA, there was a consistent trend for tumour number to decrease with folic acid or methionine supplementation on their own and increase when combined together. In a separate analysis leaving out the combination treatment, the effect of folic acid and methionine was significant (p value = 0.025, FDR = 0.042 for NWD+FA, p value = 0.042, FDR = 0.042 for NWD+M), as shown in Figure 6.10. Table 6.9 shows results of Two Way ANOVA analysis looking at the interaction of folic acid and methionine supplementation on tumour

incidence. Results showed that there was a significant interaction between folic acid and methionine supplementation on total tumours (p value = 0.005), tumours in the colon (p value = 0.027), tumours in the small intestine (p value = 0.009), tumours in mid jejunum (p value = 0.020) and tumours in the ileum (p value = 0.005). The data was further analysis by gender and showed no significant difference on total tumour number between diet groups in male (p value = 0.076, FDR = 0.759 – 1.000) and female mice (p value = 0.142, FDR = 0.539 – 1.000).

Figure 6.11 represents the average total tumour load recorded in the intestine (both colon and small intestine) of $Apc^{Min/+}$ mice for all diet treatment groups. There was no significant difference observed between all groups, with p value = 0.549 - 1.00, FDR = 1.000. Two Way ANOVA results indicate an interaction between folic acid and methionine supplementation on total tumour load which was close to statistical significance (p value = 0.055) as shown in Table 6.10. Further analysis by gender showed no significant difference on tumour load between diet groups in male (p value = 0.195, FDR = 0.918 – 1.000) and female mice (p value = 0.336, FDR = 1.000).

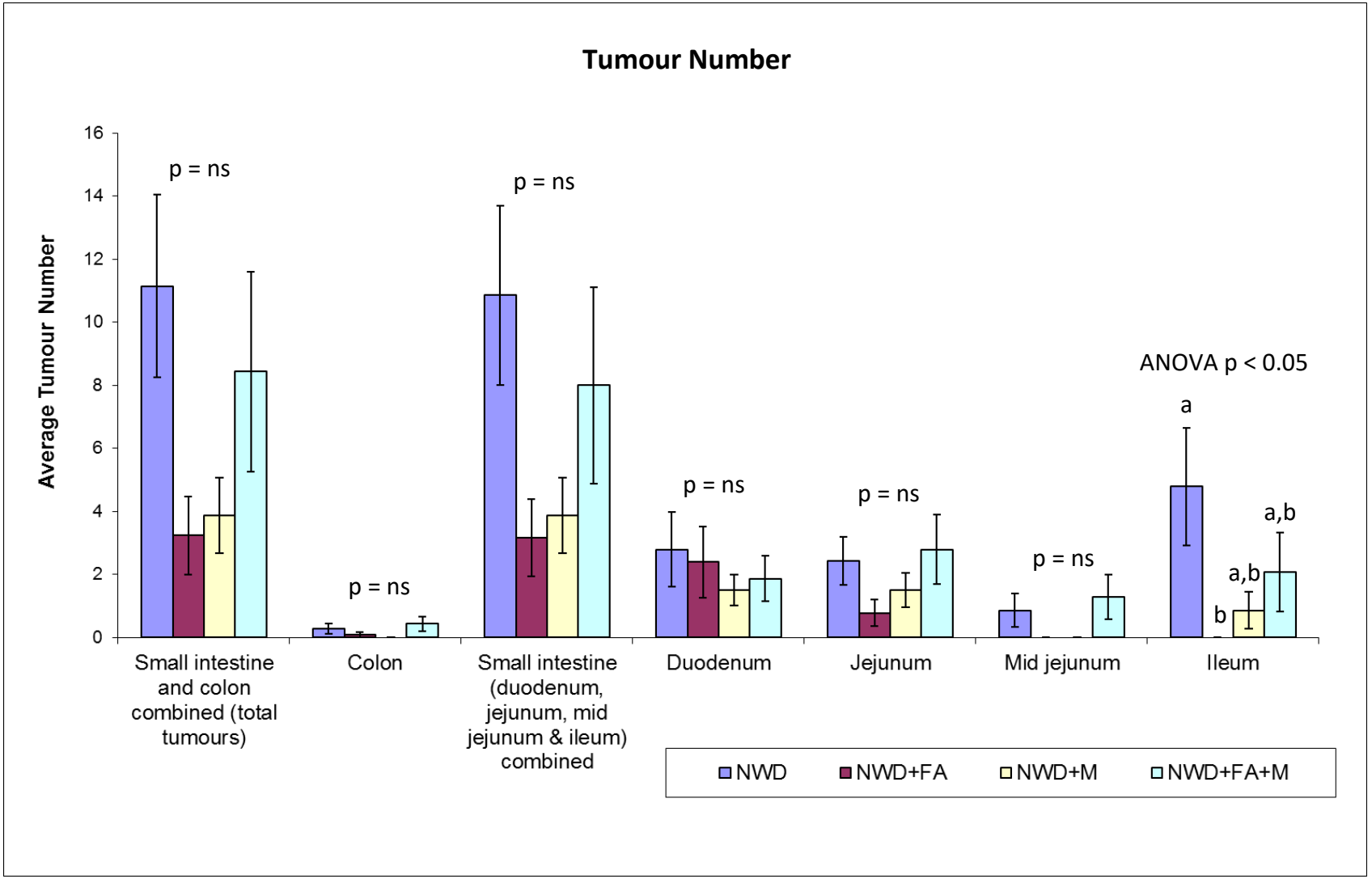


Figure 6.9: Average intestinal tumour number in $Apc^{Min/+}$ mice. Data expressed as mean ± SE.

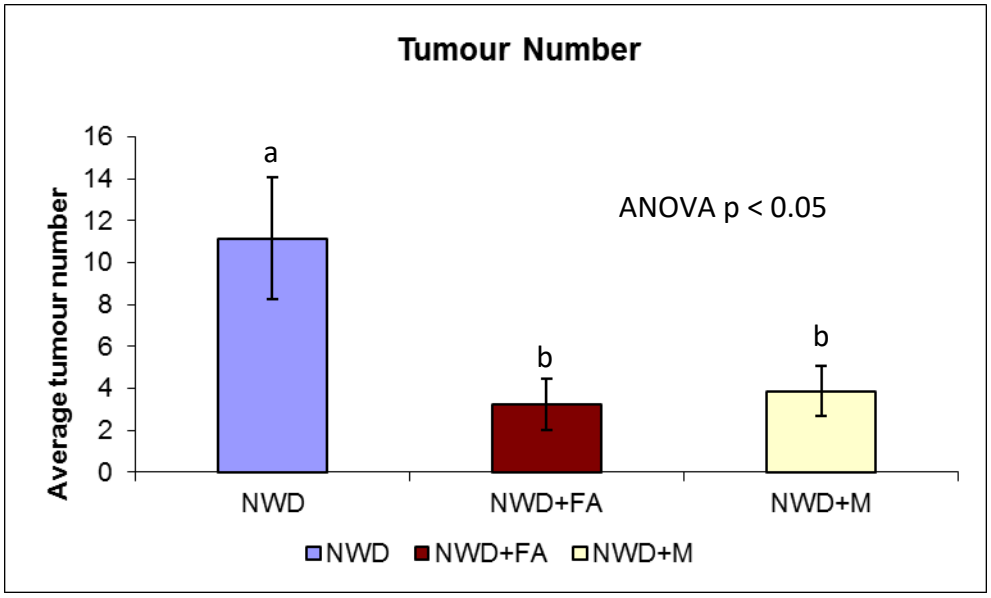


Figure 6.10: Average intestinal tumour number in $Apc^{Min/+}$ mice, leaving out NA+FA+M treatment data. Data expressed as mean ± SE.

Table 6.9: Two Way ANOVA results for folic acid and methionine interaction on tumour incidence.

Outcome	Source of Variation	P value
Total tumours (small intestine and colon combined)	Folic acid	0.233
	Methionine	0.587
	Folic acid and Methionine	0.005*
Tumours in the colon	Folic acid	0.454
	Methionine	0.919
	Folic acid and Methionine	0.027*
Tumours in the small intestine (duodenum, jejunum, mid jejunum and ileum combined)	Folic acid	0.207
	Methionine	0.571
	Folic acid and Methionine	0.009*
Tumours in duodenum	Folic acid	0.734
	Methionine	0.592
	Folic acid and Methionine	0.540
Tumours in jejunum	Folic acid	0.365
	Methionine	0.690
	Folic acid and Methionine	0.100
Tumours in mid jejunum	Folic acid	0.634
	Methionine	0.634
	Folic acid and Methionine	0.020*
Tumours in ileum	Folic acid	0.098
	Methionine	0.632
	Folic acid and Methionine	0.005*

*ANOVA $p < 0.05$

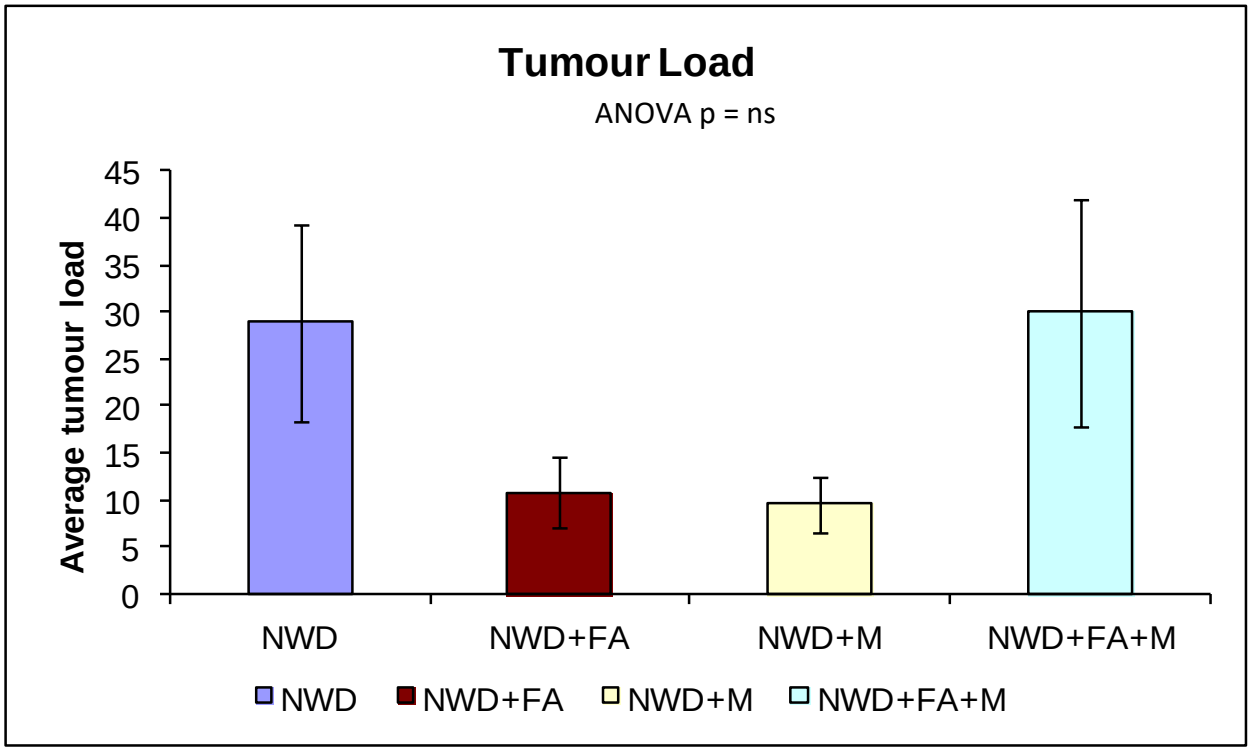


Figure 6.11: Average intestinal total tumour load in $Apc^{Min/+}$ mice. Data expressed as mean ± SE.

Table 6.10: Two Way ANOVA results for folate and methionine interaction on intestinal total tumour load.

Outcome	Source of Variation	P value
Total tumour load	Folic acid	0.811
	Methionine	0.970
	Folic acid and Methionine	0.055

6.6 Discussion

In the beginning of this study, it was hypothesised that supplementation of folic acid and methionine would reduce the risk of developing intestinal cancer in the $Apc^{Min/+}$. Results showed a reduction in average tumour number, and tumour numbers at specific locations in the intestine, with the supplementation of folic acid and/or methionine, compared to the control NWD diet. The effects of folic acid and methionine appear to be site specific in the intestine, with the site most susceptible to responding to the dietary intervention of folic acid being the ileum. Results obtained partly support the hypothesis presented in this study; supplementation of folic acid was found to significantly reduce the tumour number in the ileum, compared to control. Diet supplemented with folic acid contained 8 mg of folic acid/kg diet, equivalent to four times the basal requirement for mice. A previous study reported that dietary folate supplementation at that level significantly protects against the development of small intestinal adenomas and colon ACF, if started before the establishment of neoplastic foci in the $Apc^{Min/+}$ mice [95]. From the results in the present study, it can be observed that individual supplementation of either folic acid or methionine shows a lower number and load of tumours, compared with the effect of combined supplementation of both. The study of Finkelstein and Martin (1986) [247] showed strong changes in metabolites (increased SAM, SAH and methionine; decreased betaine and serine) in the folate/methione pathway in the liver of rats supplemented with high dose methionine. It would be interesting to test whether supplementing with folic acid in a high methionine background regulates metabolic changes induced by more methionine that may inhibit cancer growth and vice-versa.

A study by Song et al. (2000) reported similar observation of non-significant reduction of total tumour number, tumour number in the small intestine, colon, duodenum, jejunum and ileum in $Apc^{Min/+}$ mice supplemented with 8 mg folate, compared to those fed with folate restriction (0 mg folate), for the duration of 3 months study [94]. Interestingly, similar dietary intervention for 6 months showed that supplementation of 8 mg folate lead to an increase in total tumour in small intestine, tumour in duodenum, jejunum, ileum and colon. In this study, only the increase in the ileum was statistically significant, with conflicting data on the development of adenomas at this site , depending on duration of intervention [94].

Generally, in this study the $Apc^{Min/+}$ mice on folic acid restricted diet (NWD) had higher tumour number. This is consistent with epidemiological studies indicating low folate is a risk factor of colorectal cancer [25]. Similar observation was recorded by Lawrance et al. (2007), in which a diet containing 0.2–0.3 mg folic acid was associated with a higher number of adenoma in $Apc^{Min/+}$ mice, compared to a diet containing 2 mg folic acid [100]. These data are contradictory, however, with those of Trasler et al. (2003), with folate restricted diet (0 mg folate) in the $Apc^{Min/+}$ mice with mutant DNMT1 (with high baseline high baseline tumor numbers) decreased tumour numbers but higher proportion of larger tumours, compared to supplementation of basal folate level (2 mg folate) [98]. Nevertheless, in their repeat experiment, folate deficient did not affect tumor number or size in the $Apc^{Min/+}$ mice with mutant DNMT1 (with low baseline tumour number). Thus,

their results suggested that in the presence of DNMT1 deficiency, the effects of folate restriction on tumor number and size may depend on the stage of adenoma development when folate restriction is initiated.

Results obtained in this study indicated that methionine supplementation resulted in marginally lower tumour numbers recorded in $Apc^{Min/+}$ mice, compared to control diet. This is in contrast to a study by Paulsen and Alexander (2001) which showed that additional methionine at the level of 11.6 g of methionine/kg diet supplemented for 4 weeks in $Apc^{Min/+}$ mice promoted the growth of adenomas in the small intestine by increasing the surface area of tumours, whilst having no effect on the number of tumours in both the small intestine and the colon. This was compared to the standard diet containing 4.6 g of methionine/kg diet [24]. Our data may differ from their study due to these rationales; the level of methionine supplementation used in our study was higher (at 15 g of methionine/kg diet); administered for longer duration (10 weeks); earlier exposure to high methionine diet (starts at 3 weeks of age in our study, compared to 4 weeks of age in their study); and exposure to higher initial dietary methionine content (mice were fed with standard laboratory rodent diet (contains 0.59% methionine) until 3 weeks of age in our study, whereas mice in their study were fed with SDS RM3 (E) breeding diet (contains 0.34% methionine) until 4 weeks of age), thus exhibits the protecting effect of higher methionine on tumour development.

A previous study by Fan et al. (2007) showed NWD administration increased colonic tumour in $Apc^{Min/+}$ micel [233]. However, in the present study only 7 out of 55 $Apc^{Min/+}$ mice developed tumours in the colon (12.73%). This finding is consistent with previous reports which have indicated that the small intestine is the most susceptible site, and the colon is comparatively resistant to dietary manipulation in $Apc^{Min/+}$ mice [94].

As discussed in Chapter 2, it was proposed that the possible mechanisms by which dietary folate and/or methionine may affect intestinal tumour development are through the modulation of nucleotide synthesis, DNA methylation and altered polyamine metabolism [93]. At this stage, the mechanisms are remaining unclear. However results of the current study suggest that folic acid and methionine may exert their effects on tumour initiation and growth by similar mechanisms because this could not explain why supplementation with both folic acid and methionine (as in NWD+FA+M) appeared to annul the apparent protective effect of supplementing with folic acid or methionine on its own. If high folic acid inhibits MTHFR in mice as indicated by recent study [248], it might result in altered methionine metabolism in a manner that makes supplemental methionine in mice more available for polyamine synthesis and therefore stimulate tumour growth. This could be one of the explanations for the unexpected result in NWD+FA+M group. Further analyses on these samples were conducted; these data are detailed in the following chapter.

In the $Apc^{Min/+}$ mice, the onset of cancer and associated anaemia starting at around age of 10 weeks could cause distress [242, 243]. At the end stage of the study, the $Apc^{Min/+}$

mice developed tumours together with bleeding from the multiple polyps; this was associated with higher incidence of anaemia [243]. Haematocrit level is used as an indicator of anaemia, with level of below 30 % considered as anaemic [249] and below 25 % as severely anaemic [250]. All groups of the $Apc^{Min/+}$ mice were anaemic at the end of study, with the group fed with NWD being severely anaemic with average haematocrit level of 19.92 ± 13.75 % (mean ± SD), and the group on NWD+FA+M diet being borderline for severe anaemia with average haematocrit level of 25.31 ± 13.55 % (mean ± SD). Interestingly, the WT mice fed with NWD, NWD+FA and NWD+FA+M were also considered anaemic with haematocrit level less than 30 %. This could be because of the high fat and low fibre contents in the NWD diet leading to constipation and possible bleeding in the intestine, thus the higher risk of anaemia. Previous studies have shown that long term supplementation of NWD (18 – 24 months) may induce colonic tumour in WT mice [136, 138]. However, addition of methionine in NWD+M improved the anaemic condition compared to control NWD diet in both genotype groups, with significantly higher haematocrit % (above 30 %) in the WT group and a non-significant increase in the $Apc^{Min/+}$.

The observation that MTSI folate was diminished in WT mice when supplemented with folic acid is puzzling given that no such effect was observed in the $Apc^{Min/+}$ mice. However technical error in the folate assay is implausible because the MTSI folate measurement for NWD diet group mice for both WT and $Apc^{Min/+}$ mice were performed together with the same assay. The unexpected finding in WT mice needs further verification and the possibility that high dietary folic acid inhibits the expression of the folic acid receptor in WT mice needs to

be tested as a possible mechanistic explanation. A recent study in mice fed a folic acid supplemented diet showed reduced MTHFR activity and reduced MTHFR protein suggesting the plausibility of inhibitory effect of folic acid or the expression of metabolism related proteins [248]. The potential limitations of the unconventional method used for collecting small intestinal tissue is acknowledged as a potential limitation and confounding factor that may have influenced folate measurement. However, the same method was used for all animals and is unlikely to explain the observed differences.

Although it was disappointing not to have the plasma folate data, the folate measurements in the intestine was considered to be more relevant to the hypothesis and these were obtained. In the case of plasma, methionine was measurable and showed the expected trend for an increase in the mice on diets with higher methionine. It is likely that a similar trend in high plasma folate would have been observed in mice on diets supplemented with folic acid.

6.6.1 Strength of study

The strength of this study are; (i) relevant mouse model, (ii) relevant diet model, and (iii) combination study of methionine and folate.

6.6.2 Weakness of study

This study may be underpowered. The average sample size used for each group was 14 mice. Based on the original work by Moser (1990), total average tumour number per

mouse is 29 ± 10 (mean ± SD) with average life span of 119 ± 31 days (mean ± SD) (mouse were killed when moribund) [85]. The sample size of 14 mice per group gives a statistical power of 82% for a one tailed test at the 5% significance level to detect a 30% decrease in the number of tumours. A 30% reduction in tumour incidence is likely to significantly reduce morbidity and mortality. The small intestinal tumour number in $Apc^{Min/+}$ mice in this study per mouse, on average, was only 11.14 ± 10.88 (mean ± SD) and although reduction of greater than 30% was observed in NWD+FA and NWD+M relative to NWD diet group statistical significance was not achieved. This may be due to a high variability between mice and the effect of multiple comparisons in reducing statistical power.

In this study, mechanistic protein or gene expression data was not obtained. However further analyses on tissues regarding genome instability was performed (in following chapter).

6.6.3 Conclusion

The results of the present study showed a trend for tumour number to decline with folic acid or methionine supplementation on their own, which was however reversed when combined together with significant interaction being evident. The results indicate that combination effect can be opposite to single effects.

Chapter 7:

In Vivo Studies – II

The Effect of Folic Acid and Methionine on Genomic Instability in the $Apc^{Min/+}$ Mouse Model

7.1 Objective

This study was carried out to investigate the effect of supplementing a western-style diet with dietary folic acid and/or methionine on genomic instability in the $Apc^{Min/+}$ mouse model.

7.2 Hypothesis

2. Dietary folic acid and methionine promote DNA stability in the $Apc^{Min/+}$ mouse model.

7.3 Introduction

Inadequate dietary levels of nutrients required in DNA metabolism result in genomic instability [251]. Folate and methionine are two essential nutrients for maintenance of genome stability, attributable to their role as methyl donors in one-carbon metabolism impacting DNA synthesis, repair, and methylation processes, as well as polyamine synthesis (as discussed in Chapters 2 and 5). Folate is important for the synthesis of dTMP from dUMP, and for the *de novo* synthesis of methionine from homocysteine. Methionine and

folate metabolism interact in the conversion of homocysteine to methionine (as shown in Figure 2.1) [48].

Deficiency of folate reduces availability of methyl groups leading to higher cellular dUMP:dTTP ratio, thus resulting in uracil incorporation into DNA. Uracil is excised from DNA by uracil DNA glycosylase and apyrimidinic endonuclease. Extensive uracil excision by BER processes when folate is deficient can lead to DNA strand breaks and chromosome instability [49]. High uracil level and elevated micronucleus frequency (a measure of chromosome breaks) caused by methyl donor deficiency are reversible with adequate folate supply [252].

Folate and methionine may also affect DNA methylation [207, 208] due to their roles in the supply of methyl groups required by DNA methyltransferase enzymes to perform their function [209]. DNA methylation relies on the availability of methionine-derived SAM. Alterations in DNA methylation, which are also associated with DNA methyltransferase dysfunction, can lead to silencing of tumour-suppressor genes, activation of oncogenes that are usually silenced, and chromosomal instability. These are common epigenetic changes observed in cancers [253, 254]. Emerging evidence has also shown a possible relationship of dietary nutrient levels in affecting epigenetic regulatory role of DNA methylation on telomere length [212-214].

The effect of excess folic acid or methionine or their combination on genomic instability remain unclear but it is important to resolve given the trend of increased supplementation of folic acid and the use of higher protein diets involving fresh food rich in methionine. This present study was carried out to examine the effects of supplementing a western-style diet with dietary folic acid and/or methionine on genomic instability in the $Apc^{Min/+}$ mouse model. To achieve the objective, three parameters were used as indicators of genome stability; telomere length, global DNA methylation, and micronucleated erythrocytes. With respect to the latter, chromosome damage and breakage in erythroid precursor cells in the bone marrow can lead to formation of micronucleated erythrocytes [255]. The presence of micronuclei is caused by a lagging whole chromosome, or acentric chromosome fragments at anaphase during mitosis [256].

7.4 Material and Methods

7.4.1 Mouse Model and Dietary Regimen

Mouse model and dietary regimen used were as detailed in section 6.4. A total number of 113 mice (55 $APC^{Min/+}$ mice and 58 WT mice) were randomised to receive one of the four diet treatments; New Western Diet (NWD) containing baseline levels of 0.23 mg of folic acid/kg diet and 4.6 g of methionine/kg diet as control diet, New Western Diet with additional folic acid (NWD+FA) containing 8 mg of folic acid/kg diet and 4.6 g of methionine/kg diet, New Western Diet with additional methionine (NWD+M) containing 0.23 mg of folic acid/kg diet and 15 g of methionine/kg diet, and New Western Diet with folate

and methionine (NWD+FA+M) containing 8 mg of folic acid/kg diet and 15 g of methionine/kg diet from weaning (age 3 week). All mice were sacrificed when aged 13 weeks.

7.4.2 Study Design

The ability of increased dietary folic acid and/or methionine intake in the $Apc^{Min/+}$ model to promote DNA stability when administered at age 3 week at weaning until 13 week were assessed. Comparative experiments were carried out in age-matched and gender-matched WT littermates, as healthy control. At 13 weeks, blood and colon tissue were collected from all mice. Blood was used for micronucleated erythrocyte assay, whereas the colon tissue was used for telomere length and DNA methylation experiments, as summarized in Figure 7.1.

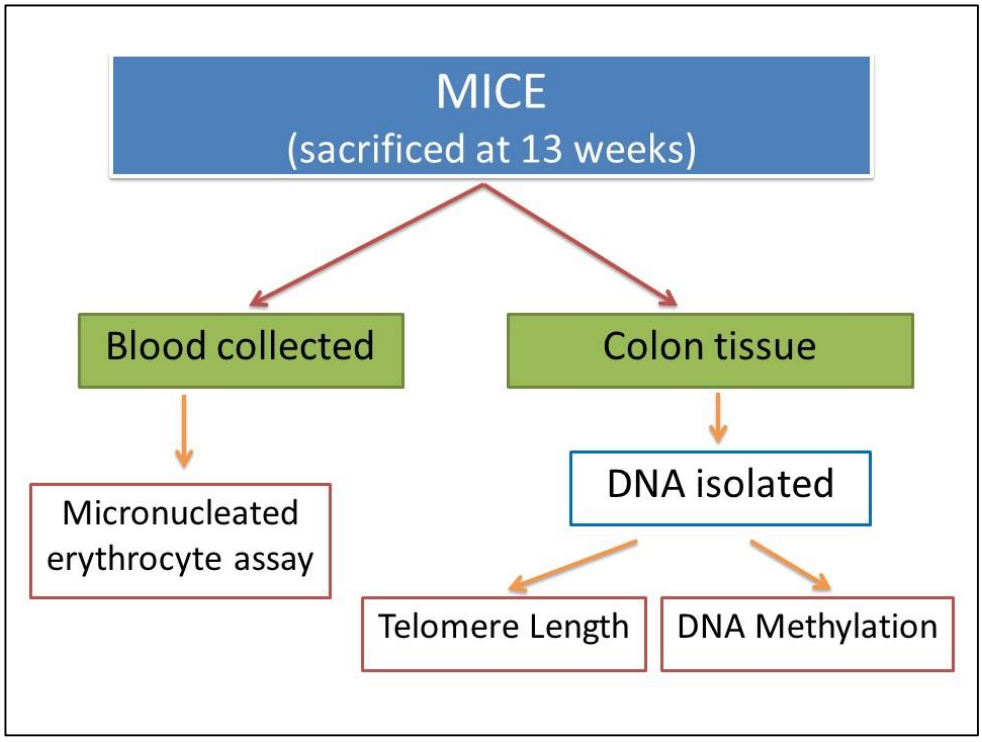


Figure 7.1: Study design to determine the effect of supplementing a western-style diet with dietary folic acid and/or methionine on genomic instability in WT and $Apc^{Min/+}$ mice.

7.4.3 Blood Collection for Whole Blood Micronucleus Erythrocyte Assay

All mice were sacrificed at age 13 weeks. Blood was collected by cardiac puncture and posterior vena cava. The blood was collected into a heparinised blood collection microtainer tube (BD, Australia) to prevent blood coagulation. The blood sample was then thoroughly mixed to prevent clotting. In a biosafety fume cabinet, 30 µLblood was pipetted onto a slide. A blood film was prepared by running a coverslip along the length of the slide. Two slides were prepared for each animal. The slides were air dried for 10 minutes. The slides were then fixed in methanol for 10 minutes. Slides were stored at room temperature in a light-proof vertical slide holding container, with desiccant, before staining.

7.4.3.1 Acridine Orange Staining

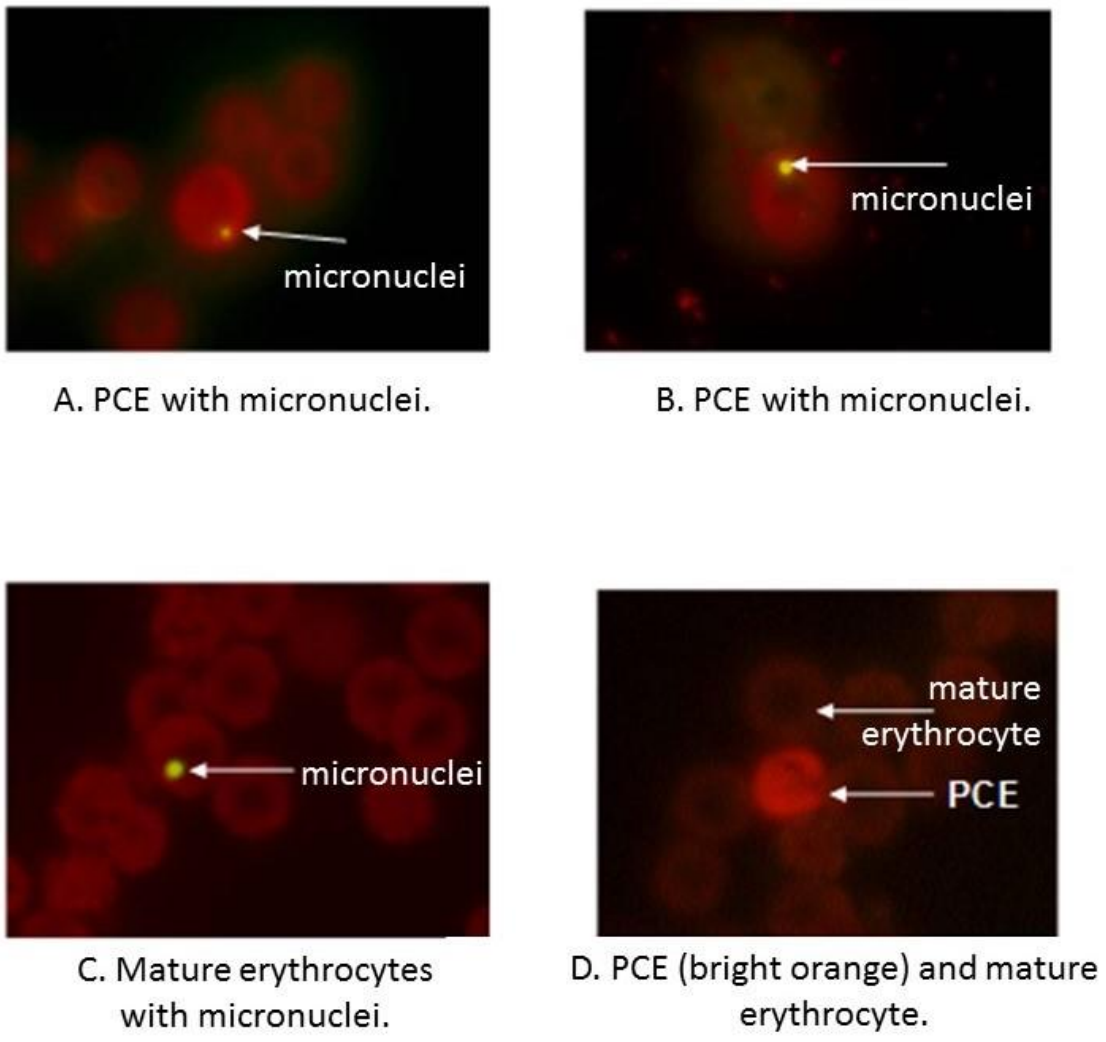
Acridine orange fluorescent staining technique was used to evaluate the micronucleus frequency in erythrocyte cells, which provides sensitive, convenient and definite quantification [257-259]. A stock solution of acridine orange (Sigma, USA) was prepared in phosphate buffer saline (PBS) at a concentration of 10 mg/mL. A 1:250 dilution was prepared in 4 X SSC (Sigma, USA) to give a final concentration of 40 µg/mL (200 µL of 10 mg/mL acridine orange in 50 mL of 4 X SSC). Slides were stained for 4 minutes, rinsed briefly in 2 X SSC and coverslipped while still wet prior to analysis using a fluorescent microscope.

Immature erythrocytes i.e. polychromatic erythrocytes (PCE) were identified by their orange-red color (Figure 7.2 D), mature erythrocytes by their green-brown color (Figure 7.2 D) and micronuclei by their yellow color (Figure 7.2 A, B, C).

The frequency of these were determined as follows:

- the frequency of PCE observed within 1000 normochromatic or non polychromatic erythrocytes (NCE) scored (PCE/1000 NCE),
- the frequency of micronucleated PCE (MN-PCE) per 100 PCE scored (MN-PCE/100 PCE), and
- the frequency of micronucleated NCE (MN-NCE) per 1000 NCE scored (MN-NCE/1000 NCE).

Figure 7.2 outlines the different cell types scored in the assay [260].



A. PCE with micronuclei.

B. PCE with micronuclei.

C. Mature erythrocytes with micronuclei.

D. PCE (bright orange) and mature erythrocyte.

Figure 7.2: Micronuclei in mature and immature erythrocyte [260]. A and B showing immature PCE with micronucleus, C: mature erythrocyte with micronucleus and D: PCE and mature erythrocyte without micronuclei in the same field view. Cells were stained with acridine orange, viewed under x1000 magnification [260].

7.4.4 DNA Isolation from the Colon Tissue

Isolation of DNA from the colon tissue was carried out using DNeasy Blood and Tissue Kit 250 (Qiagen, Melbourne, Australia). Manufacturer's protocol was followed was followed with minor modification as mentioned in [216]; ie. to minimize DNA oxidative damage, all solutions used were purged with nitrogen and supplemented with 50 µM phenyl-tert-butyl nitrone (Sigma, St. Louis, MO, USA), prior to DNA isolation process [217].

A 1 cm section of the colon was cut and placed into a 1.5 mL microcentrifuge tube. 180 µl Buffer ATL was added followed by 20 µL proteinase K (600 mAU/mL). The mixture was then incubated at 37°C for 6 hours to completely lyse the tissue. 200 µL Buffer AL was added followed by 200 µL ethanol. The mixture was mixed thoroughly using vortex. The mixture was pipetted into spin column placed in a 2 mL collection tube and centrifuged at 8000 rpm for 1 minute. The flow through and collection tube were discarded. The spin column was then placed in a new 2 mL collection tube. 500 µL Buffer AW2 (wash buffer) was added to the column. The mixture in the spin column was centrifuged at 14000 rpm for 3 minute, flow through discarded, and the column placed in a new microcentrifuge tube. 200 µL Buffer AE (elution buffer) was added directly in to the column, incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 minute. The eluate was then pipetted back into the spin column, incubated at room temperature for 1 minute and centrifuged again at 8000 rpm for 2 minutes. The final volume of approximately 200 µL of DNA concentrate was obtained. The concentration of DNA for each sample was determined with 260/280 nm measurement using NanoDrop (Biolab, ND 1000). The ratio of absorbance

at 260 nm and 280 nm is used to assess the purity of DNA. Two readings were recorded for each sample and averaged.

7.4.5 Telomere Length Assay

7.4.5.1 Quantitative Real-time Polymerase Chain Reaction (qPCR) for Telomere Length

Absolute telomere length of all DNA samples was measured with qPCR following the method described by O'Callaghan et al [216], as described in Chapter 5 (refer to 5.4.4.1).

7.4.5.2 qPCR for Rodent 36B4 Single Copy Gene (SCG)

36B4 (for rodent species) SCG was measured with qPCR following the method described in [216], as detailed in Chapter 5 (refer to 5.4.4.2).

7.4.5.3 Calculation of Telomere Length

Values of kb/reaction for telomere and genome copies/reaction for SCG were recorded. The kb/reaction value was then used to calculate total telomere length in kb per rodent diploid genome. The telomere kb per reaction value was divided by diploid genome copy number (calculated from the 36B4 Cq and standard curve) to give a total telomere length in kb per rodent diploid genome.

7.4.6 DNA Methylation Assay

Global DNA methylation was measured using the MethylFlash Methylated DNA Quantification Kit (Colorimetric) (Epigentek, USA), following the manufacturer's recommended protocol, as described in Chapter 5 (refer to 5.4.6). Results were expressed as 5-methylcytosine percentage.

7.4.7 Statistical Analyses

All data were analysed with IBM SPSS Statistic 21. One Way ANOVA with Post Hoc Multiple Comparison Bonferroni test was used to determine the significant differences between groups for all measurement. False Discovery Rate (FDR) calculation was performed when multiple comparisons were tested for statistical significance [246]. Bivariate Pearson Correlation analysis was done to determine correlation between telomere length and global DNA methylation. Two-way ANOVA analysis was carried out to determine the interaction of folic acid and methionine supplementation, as well as interaction between diet and genotype. P values of < 0.05 were considered to be significant.

7.5 Results

7.5.1 Whole Blood Micronucleus Erythrocyte Assay

Results for the whole blood micronucleus erythrocyte assay are presented in full in Table 7.1, and summarised in the text below.

7.5.1.1 Ratio of Polychromatic Erythrocytes (PCE) to Non Polychromatic Erythrocytes (NCE)

In WT mice, there was no significant difference observed between diet groups with respect to the frequency of PCE observed within 1000 NCE scored (ANOVA p value between groups = 0.067, FDR = 0.312 – 1.000), as shown in Table 7.1. Supplementation of folic acid and/or methionine tended to increase PCE frequency.

In the $Apc^{Min/+}$ mice, there was no significant difference observed between the diet groups (ANOVA p value between groups = 0.366, FDR = 1.000). In contrast to WT groups, supplementation with folic acid and/or methionine tended to decrease PCE frequency in $Apc^{Min/+}$ groups. When comparing both genotypes, it was found that the frequency of PCE was significantly higher in $Apc^{Min/+}$ compared to WT (p value = 0.000).

Based on Two Way ANOVA analysis (Table 7.2), there was no significant interaction between folic acid and methionine on PCE frequency. Two-way ANOVA results (Table 7.3) looking at the interaction of diet and genotype indicated that genotype had significant effect on PCE frequency (p value = 0.000) with higher frequency observed in the $Apc^{Min/+}$

compared to WT mice, and there was a significant interaction between methionine supplementation and genotype on PCE frequency (p value = 0.019).

7.5.1.2 Micronucleated Non Polychromatic Erythrocytes (MN-NCE)

In WT mice, there was a significant difference observed in the frequency of MN-NCE/1000 NCE scored, between diet groups (ANOVA p value between groups = 0.018, FDR = 0.033 – 1.000), as shown in Table 7.1. Group supplemented with excess methionine (NWD+M) had significantly higher MN-NCE compared the group fed with NWD+FA+M (p value = 0.011, FDR = 0.033). In the $Apc^{Min/+}$ groups, no significant difference occurred between diet groups (ANOVA p value between groups = 0.145, FDR = 0.843 – 1.000). From the results, it can be seen that methionine supplementation on its own tended to increase the frequency of MN-NCE in the WT and $Apc^{Min/+}$ mice (Table 7.1). Both WT and $APC^{Min/+}$ mice showed a reduction in MN in NCEs on NWD+FA+M related to NWD+M however this only achieved statistical significance in WT because of a large SD in $APC^{Min/+}$ mice due to two outliers.

In WT mice, Two Way ANOVA analysis for folic acid and methionine interaction on MN-NCE frequency (as shown in Table 7.2) indicated that folic acid supplementation had a significant decreasing effect (p value = 0.010). There was also a significant interaction between folic acid and methionine (p value = 0.028) on decreasing MN-NCE frequency. There was no significant interaction between diet and genotype on MN-NCE, as shown in Two-way ANOVA results in Table 7.3.

7.5.1.3 Micronucleated Polychromatic Erythrocytes (MN-PCE)

In WT groups, there were no significant differences observed in the frequency of MN-PCE per 100 PCE scored between diet groups (ANOVA p value between groups = 0.159, FDR = 1.000), as shown in Table 7.1. Similar results were observed in the $Apc^{Min/+}$ groups, with no significant difference occurred between diet groups (ANOVA p value between groups = 0.504, FDR = 1.000). Frequencies of MN-PCE tended to be higher in $Apc^{Min/+}$ mice relative to the WT group except for the NWD+FA group (Table 7.1).

Table 7.2 displays results of Two Way ANOVA analysis for folic acid and methionine interaction on MN-PCE frequency. The results indicated that and there was a significant interaction between folic acid and methionine (p value = 0.030) on decreasing MN-PCE frequency in WT mice. There was no significant interaction between diet and genotype on MN-PCE, as shown in Two-way ANOVA results in Table 7.3.

Table 7.1: Frequency of PCE observed within 1000 NCE scored, frequency of MN-PCE per 100 PCE scored and frequency of MN-NCE per 1000 NCE scored in WT and $Apc^{Min/+}$ mice (n = 113 mice; 55 $APC^{Min/+}$ and 58 WT).

	NWD	**NWD+FA**	**NWD+M**	**NWD+FA+M**	**One Way ANOVA p value between groups**
WT					
PCE/1000 NCE	61.79 ± 27.72	67.36 ± 26.36	99.14 ± 43.92	67.00 ± 54.59	0.067
MN-PCE/100 PCE	0.28 ± 0.71	0.54 ± 0.95	0.69 ± 0.78	0.13 ± 0.33	0.159
MN-NCE/1000 NCE	6.07 ± 3.54[a,b]	5.71 ± 2.13[a,b]	8.29 ± 4.41[a]	4.20 ± 0.76[b]	0.018*
$Apc^{Min/+}$					
PCE/ 1000 NCE	298.71 ± 179.79	235.54 ± 136.31	198.23 ± 95.82	224.54 ± 175.74	0.366
MN-PCE/ 100 PCE	3.23 ± 10.31	0.44 ± 0.55	1.35 ± 2.13	0.48 ± 0.56	0.504
MN-NCE/ 1000 NCE	3.57 ± 2.62	5.85 ± 5.52	14.38 ± 24.61	5.38 ± 4.94	0.145

Data expressed as mean ± SD.

*ANOVA $p < 0.05$

Groups sharing the same letter within a row are not significantly different.

Groups not sharing the same letter within a row are significantly different.

Table 7.2: Two Way ANOVA results for folic acid and methionine interaction on frequency of PCE, frequency of MN-PCE and frequency of MN-NCE scored.

Outcome	Source of Variation	P value
WT		
Frequency of PCE	Folic acid	0.218
	Methionine	0.089
	Folic acid and Methionine	0.083
Frequency of MN-PCE	Folic acid	0.419
	Methionine	0.978
	Folic acid and Methionine	0.030*
Frequency of MN-NCE	Folic acid	0.010*
	Methionine	0.806
	Folic acid and Methionine	0.028*
$Apc^{Min/+}$		
Frequency of PCE	Folic acid	0.660
	Methionine	0.187
	Folic acid and Methionine	0.288
Frequency of MN-PCE	Folic acid	0.216
	Methionine	0.494
	Folic acid and Methionine	0.494
Frequency of MN-NCE	Folic acid	0.379
	Methionine	0.198
	Folic acid and Methionine	0.127

*ANOVA $p < 0.05$

Table 7.3: Two Way ANOVA results for folic acid supplementation (NWD+FA), methionine supplementation (NWD+FA+M) and combined supplementation of both (NWD+FA+M), and genotype interaction, on frequency of PCE, frequency of MN-PCE and frequency of MN-NCE scored.

Outcome	Source of Variation	P value
Folic acid supplementation (+FA)		
Frequency of PCE	Folic acid supplementation	0.353
	Genotype	0.000*
	Folic acid supplementation and genotype	0.269
Frequency of MN-PCE	Folic acid supplementation	0.377
	Genotype	0.318
	Folic acid supplementation and genotype	0.285
Frequency of MN-NCE	Folic acid supplementation	0.334
	Genotype	0.234
	Folic acid supplementation and genotype	0.187
Methionine supplementation (+M)		
Frequency of PCE	Methionine supplementation	0.272
	Genotype	0.000*
	Methionine supplementation and genotype	0.019*
Frequency of MN-PCE	Methionine supplementation	0.582
	Genotype	0.219
	Methionine supplementation and genotype	0.400

Frequency of MN-NCE	Methionine supplementation	0.075
	Genotype	0.699
	Methionine supplementation and genotype	0.258
Combined supplementation (+FA+M)		
Frequency of PCE	Combined supplementation	0.316
	Genotype	0.000*
	Combined supplementation and genotype	0.249
Frequency of MN-PCE	Combined supplementation	0.275
	Genotype	0.225
	Combined supplementation and genotype	0.330
Frequency of MN-NCE	Combined supplementation	0.713
	Genotype	0.454
	Combined supplementation and genotype	0.067

*ANOVA $p < 0.05$

7.5.2 Telomere Length

Results for telomere length in the intestinal tissue are displayed in Figure 7.3. From the results obtained, there were no significant effects of supplementation with dietary folic acid and/or methionine in the WT (p value = 1.000, FDR = 0.384 – 1.000) and $Apc^{Min/+}$ mice (p value = 1.000, FDR = 1.000), compared to groups fed only control NWD diet. From the data, it can be seen that supplementation of folic acid in NWD+FA tended to decrease telomere length in WT, but increased telomere length in $Apc^{Min/+}$ mice. Combined supplementation with folic acid and methionine in NWD+FA+M tended to increase telomere length in WT, and in contrast decreased telomere length in $Apc^{Min/+}$ groups (non-significantly).

Two-way ANOVA looking at the interaction of folic acid and methionine indicated that neither folic acid nor methionine had a significant effect on telomere length in WT (p value = 0.975 and 0.189, respectively), or $Apc^{Min/+}$ (p value = 0.934 and 0.318, respectively), (Table 7.4). There was a statistically marginal significant interaction between folic acid and methionine found in WT mice (p value = 0.054). There was no significant interaction between folic acid and methionine found (p value = 0.299) in $Apc^{Min/+}$mice. Two-way ANOVA looking at the interaction of diet and genotype indicated no significant interaction on telomere length (Table 7.5).

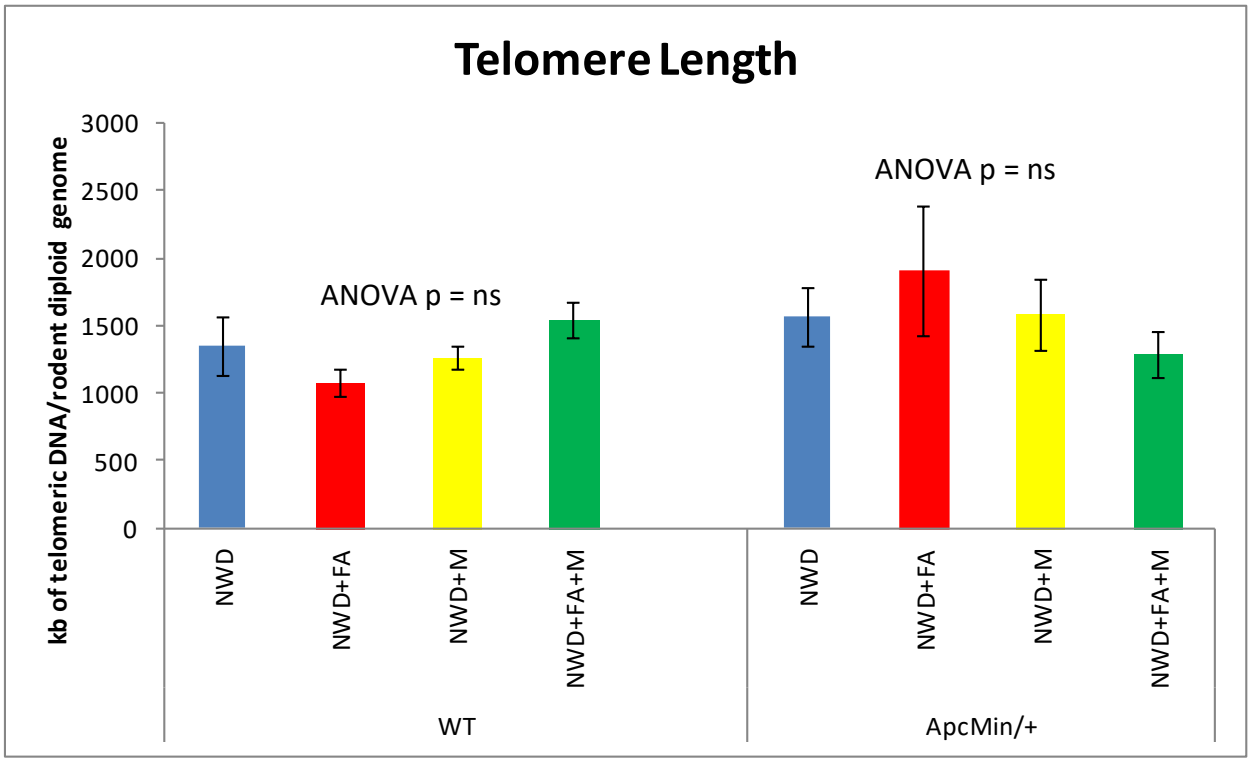


Figure 7.3: Average telomere length (expressed as kb of telomeric DNA per rodent diploid genome) in colon tissue of the WT and $Apc^{Min/+}$ mice. Data expressed as mean ± SE.

Table 7.4: Two Way ANOVA results for folic acid and methionine interaction on telomere length in colon tissue.

Outcome	Source of Variation	P value
Telomere length in WT	Folic acid	0.975
	Methionine	0.189
	Folic acid and Methionine	0.054*
Telomere length in $Apc^{Min/+}$	Folic acid	0.934
	Methionine	0.318
	Folic acid and Methionine	0.299

*ANOVA $p < 0.05$

Table 7.5: Two Way ANOVA results for folic acid supplementation (NWD+FA), methionine supplementation (NWD+FA+M) and combined supplementation of both (NWD+FA+M) and genotype interaction on telomere length in colon tissue.

Outcome	Source of Variation	P value
Folic acid supplementation (+FA)		
Telomere length	Folic acid supplementation	0.908
	Genotype	0.068
	Folic acid supplementation and genotype	0.280
Methionine supplementation (+M)		
Telomere length	Methionine supplementation	0.851
	Genotype	0.196
	Methionine supplementation and genotype	0.803
Combined supplementation (+FA+M)		
Telomere length	Combined supplementation	0.819
	Genotype	0.924
	Combined supplementation and genotype	0.204

7.5.3 DNA Methylation

Figure 7.4 shows the results for global DNA methylation (5-methylcytosine percentage) in colon tissue of the WT and $Apc^{Min/+}$ mice for all dietary treatments. There were no significant differences observed with folic acid and/or methionine supplementation in the WT (multiple comparison p value = 0.533 - 1.000, FDR = 1.000) and $Apc^{Min/+}$ mice (p value = 0.186 - 1.000, FDR = 0.558 – 1.000), compared to groups fed the control NWD diet. In both WT and $Apc^{Min/+}$ groups, supplementation of either folic acid or methionine (in NWD+FA or NWD+M) (non-significantly) decreased global DNA methylation.

Two-way ANOVA indicated that there was a significant interaction between folic acid and methionine on global DNA methylation in $Apc^{Min/+}$ mice (p value = 0.002), and approached significance in WT mice (p value = 0.062), as shown in Table 7.6. Two-way ANOVA looking at the interaction of diet and genotype (Table 7.7) showed significant effects of folic acid or methionine supplementation on global DNA methylation (p value = 0.005 and 0.014, respectively). Supplementation of either folic acid or methionine tended to decrease global DNA methylation in both genotypes.

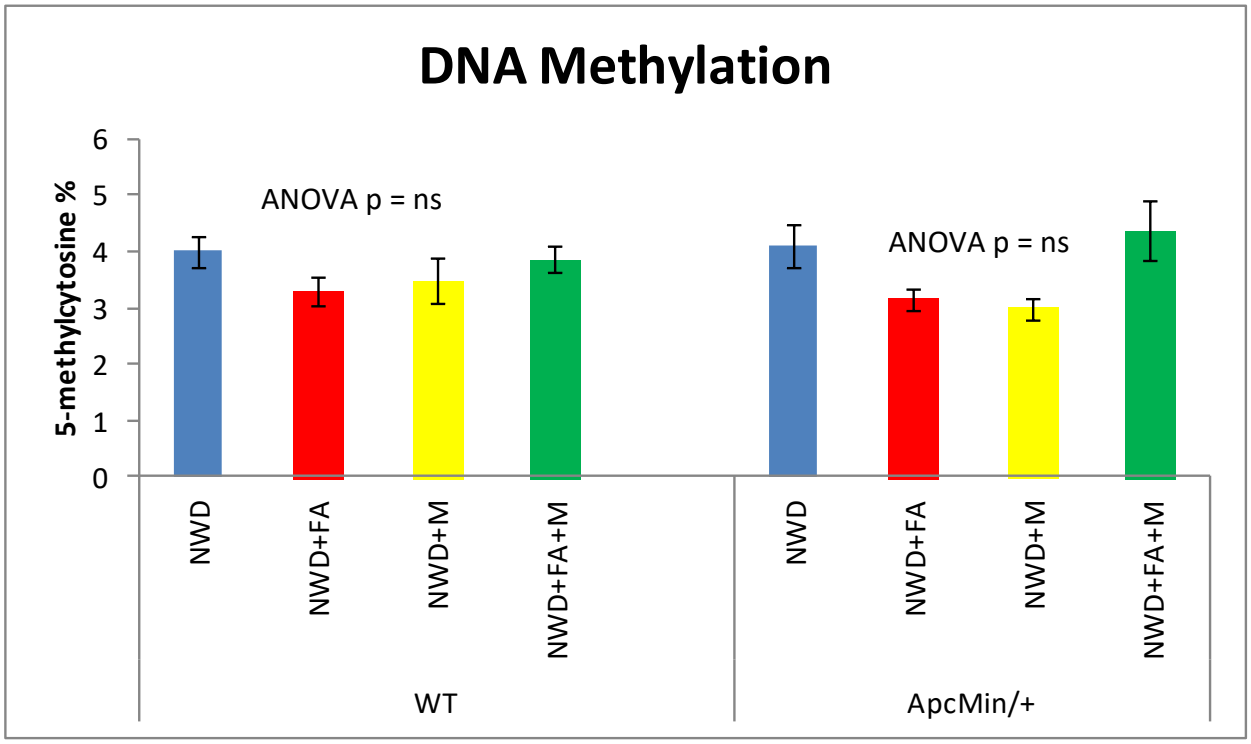


Figure 7.4: Global DNA methylation (expressed as percentage of 5-methylcytosine) in colon tissue of the WT and $Apc^{Min/+}$ mice. Data expressed as mean ± SE.

Table 7.6: Two Way ANOVA results for folic acid and methionine interaction on DNA methylation in colon tissue.

Outcome	Source of Variation	P value
DNA methylation in WT	Folic acid	0.565
	Methionine	0.946
	Folic acid and Methionine	0.062
DNA methylation in $Apc^{Min/+}$	Folic acid	0.530
	Methionine	0.880
	Folic acid and Methionine	0.002*

*ANOVA $p < 0.05$

Table 7.7: Two Way ANOVA results for folic acid supplementation (NWD+FA), methionine supplementation (NWD+FA+M) and combined supplementation of both (NWD+FA+M) and genotype interaction on DNA methylation in colon tissue.

Outcome	Source of Variation	P value
Folic acid supplementation (+FA)		
DNA methylation	Folic acid supplementation	0.005*
	Genotype	0.903
	Folic acid supplementation and genotype	0.695
Methionine supplementation (+M)		
DNA methylation	Methionine supplementation	0.014*
	Genotype	0.510
	Methionine supplementation and genotype	0.371
Combined supplementation (+FA+M)		
DNA methylation	Combined supplementation	0.856
	Genotype	0.428
	Combined supplementation and genotype	0.560

*ANOVA $p < 0.05$

7.5.4 Correlation

There were no significant relationship observed; between telomere length and DNA methylation status in WT groups (p value = 0.482, Pearson correlation = 0.094); in the $Apc^{Min/+}$ groups (p value = 0.263, Pearson correlation = -0.153); and when data from all WT and $Apc^{Min/+}$ mice were combined (p value = 0.402 Pearson correlation = -0.080).

Table 7.8 displays results of Pearson correlation analysis between tumour number and tumour load with genome instability measurements in this study. There were no significant correlations observed.

Table 7.8: Pearson correlation analysis between tumour incidence or load and genome instability.

Outcome of tumour measurement	Genome instability measurement	R value	P value
Tumour number	MN-NCE	-0.203	0.138
	MN-PCE	-0.050	0.715
	Telomere length	-0.086	0.534
	DNA methylation	0.074	0.589
Tumour load	MN-NCE	-0.204	0.135
	MN-PCE	0.223	0.102
	Telomere length	-0.093	0.500
	DNA methylation	0.033	0.811

7.6 Discussion

It was hypothesized that supplementation of folic acid and/or methionine would promote DNA stability in the $Apc^{Min/+}$ mouse model, with hypotheses that folic acid and/or methionine deficiency would increase telomere length and global DNA methylation, while reducing micronucleus frequency. Results from the whole blood micronucleus erythrocyte assay indicated that there were no changes on chromosomal damages induced by increased dietary folic acid and/or methionine intake in the $Apc^{Min/+}$ model, but in WT mice there was a significant interaction effect of methionine and folic acid supplementation in reducing micronucleus frequency in NCEs and PCEs (Table 7.1 and 7.2). The results suggest that increasing combined folic acid and methionine levels at the concentrations used in this study protected against chromosome damage in WT mice but not $Apc^{Min/+}$ mice. One of the limitations in this study is the possibility that it may have been underpowered for some of the primary biomarkers such as MN frequency in PCE due to low numbers of cells analysed. It is recommended to score 2000 PCE to detect treatment-induced increases in the MN-PCE frequency to minimize the standard deviation [261]. In the current study, PCE scored for MN frequency were limited to PCE observed within 1000 NCE scored. MN, the biomarker of DNA damage used in the study could have been measured in the intestines however this was precluded by the need to use the tissue to measure folate and methionine, and to isolate DNA for methylation and telomere measurements. Nevertheless, some cells could have been spared for MN measurement which would have strengthened the study.

Swayne et al. (2012) [256] reported two experiments conducted to examine the effects of folate on chromosome damage in female and male mice. In the first experiment, male mice were given folic acid deficient diet containing 0 mg folic acid/kg diet, control diet containing basal level of 2 mg folic acid/kg, or folic acid repleted containing 6 mg folic acid/kg diet, from weaning to maturity. MN-NCE frequency was observed to be higher in group fed with folic acid deficient diet compared to other groups. In the second experiment of multigenerational study, female mice were given similar dietary regimens throughout pregnancy, lactation, and breeding for 3 generations and the male mice produced from the third generation were fed the same diet as their mothers from weaning. Their results showed no changes in the MN-reticulocytes and MN-NCE frequency percentage in the folic acid repleted (6 mg folic acid/kg diet) mice compared to control (2 mg folic acid/kg diet) and folic acid deficient (0 mg folic acid/kg diet) diet , indicating supplemental dietary folic acid has no significant effect on chromosome damage in erythrocyte progenitor cells of mice [256].

Previous study by Aissa et al. (2013) showed higher frequencies of micronuclei in the peripheral blood polychromatic erythrocytes after 10 weeks of dietary methionine supplemented intervention in mice, with the frequency of micronuclei in the methionine-supplemented diet increased by 122% compared with the control group [161]. In our study, there was a trend for an increase in MN in PCEs and NCEs with methionine supplementation on its own but did not achieved statistical significance. Their results demonstrated that methionine has a tissue-specific effect on chromosomal stability in adult female mice. In

their study, methionine-supplemented diet induced both chromosomal and DNA damage in peripheral blood cells whereas the methionine deficient diet reduced basal DNA damage in the liver [161].

A study by Wainfan et al. (1989) in male rats detected rapid hypomethylation of DNA and tRNA with significant elevation in liver DNA synthesis, following a diet deficient in folic acid, methionine, choline and vitamin B, observed for 4 weeks [262]. This is in agreement with an earlier study carried out by Wilson et al. (1984) in male rats who observed hypomethylation of hepatic nuclear DNA in rats fed with methyl deficient diet [263].

Another study by Bhave et al. (1988) showed that c-H-*ras* and c-K-*ras* oncogenes were hypomethylated in DNA derived from both neoplastic and preneoplastic livers of rats supplemented with methyl deficient diet containing no methionine or choline but had folic acid and vitamin B_{12} at standard levels [207]. Meanwhile a study by Kim et al. (1997) have shown that folate deficient diet induces DNA strand breaks and hypomethylation within the p53 tumor suppressor in male Sprague-Dawley rats [128].

It is proposed that methionine supplementation increasing chromosomal damage in peripheral blood cells is related to increasing homocysteine concentration, which has positive association with higher micronucleus frequency [264, 265] and inducing oxidative stress [266, 267]. However, homocysteine level was not measured in the present study.

In the present study, the frequency of PCE was significantly higher in $Apc^{Min/+}$ compared to WT, suggesting a higher cell proliferation rate [268]. This observation is supported by the $Apc^{Min/+}$ having higher cell proliferation via the effect of Apc complex in the WNT signalling pathway [88] (as shown in Figure 2.2). The increased proliferation response of erythrocyte progenitor cells may also have been a response to counteract the anaemia induced by intestinal bleeding. Intestinal bleeding was observed with the presence of faecal occult blood.

Telomere length results obtained in this study indicated no significant changes observed with alteration in folic acid and/or methionine supplementation. The finding did not support previous studies in human indicated that folate level is positively associated with telomere length [214, 221]. A study by Richards et al. (2008) in 1319 healthy subjects observed longer leukocyte telomere length in individuals with higher serum folate levels [221]. A study by Bull et al. (2009) using a cross sectional design, compared samples collected from 43 younger (aged between 18 - 32 years old) and 47 older (aged between 65 – 83 years old) South Australian adults and found a positive correlation between telomere length in peripheral blood lymphocytes and plasma folate levels in older males [214].

Nevertheless, the result in this study is in agreement with a recent study by Liu et al. (2013). Their study in a total of 1715 participants from the Nurses' Health Study (NHS) showed no significant relations between peripheral blood leukocyte telomere length and

folate or methionine levels. The latter were measured both in plasma and calculated from food frequency questionnaires (FFQ) [269].

Global DNA methylation results showed no significant changes from folic acid and/or methionine supplementation in WT or $Apc^{Min/+}$ groups. This is in agreement with previous report by Swayne et al. (2012) using similar protocol. Their results showed no significant changes of folic acid deficiency or excess on global DNA methylation [256]. In the present study, our data (Table 7.7) suggest a significant effect of folic acid or methionine supplementation on decreasing DNA methylation. This is similar to the non-significant observation reported by Swayne et al. (2012), showing a trend for decreased global DNA methylation with increased folic acid supplementation in Balb/c mice [256]. In addition, our results suggest a significant interaction of folic acid or methionine on global DNA methylation when analysed by genotype (Table 7.6).

The results of this investigation did not support the hypothesis that telomere length and DNA methylation are related to each other in tumour number or load. In conclusion, based on the results obtained in this study, there is insufficient evidence to suggest that folic acid and/or methionine supplementation substantially alter genome instability, telomere length or DNA methylation in WT or $Apc^{Min/+}$ mice fed a western diet.

Chapter 8:

Conclusion, General Discussion & Future Direction

Diet has been proven to play a crucial role in cancer development and progression. Accordingly, this research was initiated to study the potential of two important nutrients, namely folate and methionine, in reducing colorectal carcinogenesis, and their possible mechanisms of action. Previous epidemiologic and experimental studies link folate and/or methionine consumption with risk of colorectal cancer and propose that mechanisms may involve an imbalance in DNA methylation, and DNA synthesis and repair [21-23, 25, 26, 72]. This study was carried out to provide evidence to resolve the conflicting information, by investigation on the role of excess or restriction of folic acid and/or methionine on genomic instability and cancer growth, using both *in vivo* and *in vitro* approaches. It was hypothesized that (i) restriction of folic acid and/or methionine induces genomic instability and prevents excessive proliferation of colorectal cancer cells *in vitro,* and (ii) dietary folic acid and/or methionine has a protective effect on intestinal and colorectal tumorigenesis *in vivo* in the cancer prone mouse $Apc^{Min/+}$ model.

The hypothesis that folic acid or methionine deficiency prevent excessive proliferation of cancer cells was supported in the *in vitro* study using HT29 cells. In the 14 day study, HT29 cell proliferation decreased in a dose-dependent manner when cultured in folic acid- or methionine-deficient conditions (4 – 50 nM/L folic acid, 4 – 50 µM/L methionine). Supraphysiological condition of high folic acid concentration (RPMI, 2655 nM)

resulted in higher HT29 cell proliferation compared to other treatments which were deficient in folic acid ($p < 0.05$). Whereas, methionine deficiency (non-significantly) inhibited cell proliferation in HT29. The proposed mechanism involved is that higher concentration of folate resulted in higher concentration of intracellular 5'-methyltetrahydrofolate, methionine and SAM which may have stimulated polyamine synthesis which drives cell growth [27, 156]. Unfortunately, the timeframe for this work did not allow for further analyses of these factors, however, this would be an important future research direction.

Genome instability *in vitro* in HT29 cells was assessed by scoring biomarkers of DNA damage using the CBMN Cyt assay [168], quantification of absolute telomere length using qPCR [216], and global DNA methylation was quantified by measuring 5-methylcytosine . The hypothesis that excess folic acid or methionine would promote DNA stability in HT29 cells was not supported when assessed using the CBMN Cyt assay. Higher concentration of folate (RPMI, 2655nM) induced apoptosis ($p<0.05$), however, addition of methionine had no effect. Folic acid and methionine deficiency did not change indicators of genome damage measured using the CBMN Cyt assay (i.e. micronuclei, nucleoplasmic bridges, and nuclear buds). In conclusion, restriction of folic acid or methionine prevents rapid cellular proliferation, and relative to high folate, the lower folate concentration reduces apoptosis in HT29 cell lines. With regard to cancer, the findings suggest that restricting folate or methionine slow the growth of colon cancer cells without increasing chromosomal instability or DNA damage in HT29 cells. A weakness of the *in vitro* study was that results

were restricted to HT29 cells. Future studies should be aimed at comparing the effects in different colon cancer cell lines. It is also essential to validate the observations made and to test the possible mechanisms such as amplification of the folate receptor.

The hypothesis was also rejected in the telomere length experiment, because lower folic acid or methionine concentration was associated with longer telomeres in the short term (14 day) period. The hypothesis that folic acid or methionine deficiency would lead to a decrease in DNA methylation was also not supported. While no significant differences were recorded, a trend of increased DNA methylation with methionine deficiency was observed. It appears from these new data that HT29 cells may be resistant to the genome instability biomarkers investigated in this study.

Results obtained from the *in vivo* experiments using the cancer prone $Apc^{Min/+}$ mouse model partially supported the hypotheses presented in this study. The effects of folic acid and methionine appear to be site specific in the intestine. The site which was most susceptible to dietary intervention with folic acid was the ileum. Supplementation was found to significantly reduce the tumour number at this site, compared to folic acid deficient diet which resulted in higher ileal tumour number. The data also suggested that dietary supplementation with folic acid or methionine alone may be more protective compared to combined supplementation of both nutrients. Although folate and methionine are interrelated in one carbon metabolism, results obtained from this study suggest that folate and methionine may work via different mechanisms to affect tumour growth. This is

perhaps due to possible antagonistic effects of folic acid and methonine when present simultaneously at high doses in the $Apc^{Min/+}$ mice.

Genome instability *in vivo* was evaluated by whole blood micronucleus erythrocyte assay, quantification of absolute telomere length using qPCR [216], and quantification of 5-methylcytosine percentage to determine global DNA methylation. The hypothesis that excess folic acid or methionine promotes DNA stability was rejected. There were no significant changes in micronucleus frequencies (MN-NCE and MN-PCE), telomere length or global DNA methylation observed except, perhaps for an interaction effect of methionine and folic acid in promoting genome stability in erythroid progenitor cells in WT mice. It appears, however, that dietary supplementation or restriction of folic acid and/or methionine do not promote or protect against DNA instability in $Apc^{Min/+}$ mice.

One of the limitations in this study was that $Apc^{Min/+}$ mice mainly develop polyps in the small intestine whereas in humans intestinal cancer develops mainly in the colon [94]. The expectation of producing more colonic tumours by supplementing a western style diet as has been reported by Fan et al. (2007) [233], however was not observed. In this study, only 7 out of 55 $Apc^{Min/+}$ mice developed tumour in the colon (12.73%). It would be interesting to study whether an increase in colonic tumours occurs with longer duration of study, together with a larger sample size. This may allow the development and progression of tumours in the colon, in a population size powered to deliver significant results, based on our data. A study by Kadaveru et al. (2012) observed an encouraging effect of folate

deficiency, in combination with choline, methionine and vitamin B_{12} depletion, in reducing colonic tumour size in $Apc^{Min/+}$ mice with longer intervention period until 16 weeks of age [143]. Meanwhile modification to the $Apc^{Min/+}$ model through Cre expression in the intestinal tract increases colonic tumour development, therefore increasing the similarity to human condition [149-152]. This offers a more relevant $Apc^{Min/+}$ model for investigating the effects of dietary factors that may increase colorectal cancer in human.

Another limitation was that the synthetic form of folate, folic acid, was used in this study which is not the reduced and methylated form that occurs naturally in foods. Peyer's patches in the intestines were not quantified in this study but might have influenced the results obtained if folate and methionine intake affected their number. A recent study observed that the number of intestinal polyps is decreased with increased Peyer's patches in $Apc^{Min/+}$ mice [270].

The outcomes of this study narrow the knowledge gaps presented in the earlier part of this thesis, by showing some significant novel interaction effect of folate and methionine *in vivo* and enrich the data available on the role of folate and methionine in colorectal cancer initiation and progression. Hopefully this study will encourage more research to further investigate the relationship between dietary factors (especially folic acid and methionine) and colorectal cancer, to improve understanding and clinical approaches. It is also important to emphasize that genetic factors may provide further information on different aspect of folate and methionine metabolism. Further broader perspectives of

folate and methionine-dependent tumorigenesis mechanisms requires further exploration. This includes alteration in histone and DNA methylation status, evaluation of chromosomal instability and DNA damage, the modulating effect of SAM: SAH ratio, interaction between APC and polyamines, and DNA methyltransferase expression and activity [93]. At this stage, the underlying mechanisms involved remain controversial and incompletely understood.

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APPENDIX: PAPER REPRINTS

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Review

The influence of folate and methionine on intestinal tumour development in the $Apc^{Min/+}$ mouse model

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ABSTRACT

Folate and methionine are critical for one-carbon metabolism impacting DNA synthesis, repair, and methylation processes, as well as polyamine synthesis. These micronutrients have been implicated in colorectal cancer risk. There are, however, inconsistencies within the literature, with some studies showing restriction to have tumour-inhibitory effects, whereas others suggest excess to have adverse outcomes. We conducted a review of the published data to examine the accumulated evidence for involvement of dietary folate and/or methionine restriction or excess in intestinal tumour development in the $Apc^{Min/+}$ mouse model, which is genetically prone to develop such cancers. Thirteen publications were selected for evaluation based on the following inclusion criteria: (i) use of $Apc^{Min/+}$ mouse model; (ii) interventions using dietary folate and/or methionine; and (iii) primary outcome measures focused on intestinal tumour development. We found that nutritional modulation of folate and methionine was shown to have different effects on intestinal cancer in the $Apc^{Min/+}$ mouse, depending on the dosage, duration and timing of intervention, and interaction of the $Apc^{Min/+}$ genotype with other genetic factors affecting folate and DNA methylation metabolism. Although some studies showed that folate deficiency before tumorigenesis tended to increase risk of tumour formation, there are inconsistencies regarding whether excess folate post-weaning or after tumour initiation increases intestinal tumour burden. Altogether, the pooled data do not appear to indicate a difference in effect on intestinal tumour incidence between post-weaning diets that are folate deficient or folate adequate. The $Apc^{Min/+}$ mouse is a useful model for assessment of the impact of dietary folate on intestinal tumour development, but further research is required to understand the reasons for these inconsistencies amongst studies based on likely mechanisms, including modulation of nucleotide synthesis, DNA methylation, and chromosomal instability, which may affect the rate of cellular division and its control.

Contents

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1. Introduction

1.1. Background

Folate is a water-soluble vitamin that is essential for key cellular metabolic processes, and it occurs naturally in foods such as cereals and pulses [1] and green leafy vegetables [2]. In many countries, including the United States of America and Australia, the practice of mandatory folic acid fortification in wheat flour has provided an extensive range of food options that contribute to an individual's ability to meet the recommended daily requirement, which is 400 μg/day [3]. Adequate intake of folate can reduce the risk of neural tube defects, anaemia, vascular diseases, adverse pregnancy outcomes, neuropsychiatric disorders (as reviewed in [4]), atherosclerosis (as reviewed in [4,5]) and colon cancer occurrence [6,7]. Folate is essential for maintenance of genome stability due to its role as a methyl group donor for the synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP), as well as for the *de novo* synthesis of methionine from homocysteine (HCY) (refer to Fig. 1) [8]. Reduced availability of methyl groups arising from folate deficiency leads to an increase in the dUMP:dTTP ratio, resulting in increased uracil incorporation into DNA. Extensive uracil excision by base excision repair (BER) processes when folate is deficient can cause DNA strand breaks and chromosome instability [9].

A micronutrient that is metabolically related to folate is methionine. This essential amino acid is one of the key building blocks of protein, and it also has significant antioxidant properties both in its sulphur form [10] or as selenomethionine [10,11]. Good dietary sources of methionine include Brazil nuts [12], flaxseed [13] and meat [14]. Methionine is essential in the methylation process as the precursor of S-adenosyl methionine (SAM), a primary methyl-group donor (as shown in Fig. 1) [15]. It is also required for cell growth as a precursor for the aminopropyl moieties of spermidine and spermine, the key polyamines required for transcription and translation, and which stimulate normal and neoplastic cell division [16,17]. Current evidence suggests that dysfunction of the methionine cycle and/or methionine deficiency may be a contributing factor in the development of several types of cancer, including those of the liver and colon [18,19].

Fig. 1 shows a simplified diagram of the metabolic relationship between folate and methionine, under normal conditions, and the pathways by which these two nutrients interrelate in the synthesis, repair and methylation of DNA, and in the synthesis of polyamines.

Control of gene expression is effected through epigenetic processes such as methylation of cytosine residues in the DNA sequence of gene promoters. This process of DNA methylation is regulated by the ratio of SAM and S-adenosyl homocysteine (SAH) and catalysed by DNA methyltranferase enzymes, which transfer

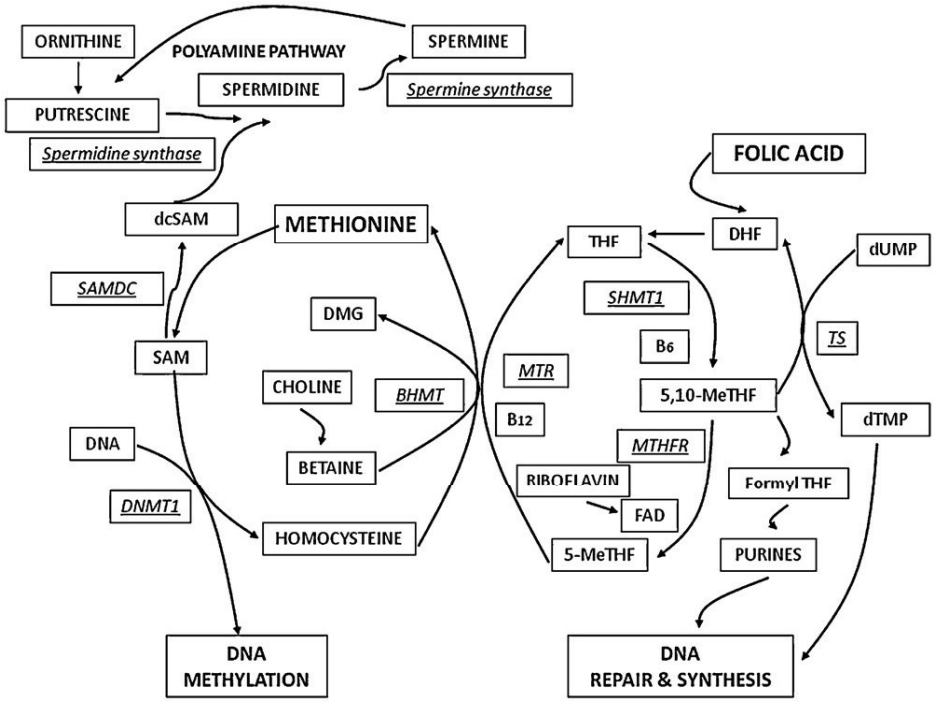

Fig. 1. The main metabolic pathways by which folate and methionine interact in the process of DNA repair, synthesis and methylation, as well as synthesis of polyamines. *Abbreviations*: B6, pyridoxine; B12, cobalamin; BHMT, betaine:homocysteine methyltransferase; DHF, dihydrofolate; DMG, dimethylglycine; FAD, flavin adenine dinucleotide; 5-MeTHF, 5-methyltetrahydrofolate; 5,10-MeTHF, 5,10-methylenetetrahydrofolate; MTR, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; DNMT1, DNA (cytosine-5-)-methyltransferase 1; SAM, S-adenosyl methionine; SHMT1, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase; SAMDC, S-adenosyl methionine decarboxylase; dcSAM, decarboxylated SAM. Enzymes are underlined.
Adapted from [8,17].

methyl groups from SAM to cytosines [20,21]. Methionine and folate interrelate in this metabolic process at the stage of conversion of homocysteine to methionine. Dysregulation of DNA methylation has been consistently associated with cancer initiation (as reviewed in [22–24]). Chemical genotoxins, which may cause mutated and/or epigenetically silenced tumour suppressor genes, are amongst the main initiators of neoplastic changes [25]. The key stages of cancer development (initiation, promotion and progression) are modulated by factors which in turn can be linked to metabolism, diet [26,27], and the environment (as reviewed in [28]). Since the landmark review by Doll and Peto [29], which was then proved by Boffetta [30], it has concluded that diet constituted a means of protecting against or promoting cancer, numerous *in vivo* (human and animal) studies have been conducted to elucidate the mechanistic link between dietary practice and tumour development.

In general, most epidemiological and experimental studies have specifically linked deficiency of dietary methyl donors, folate and methionine, with colon cancer risk (as reviewed in [31,32]). It was hypothesised that this is due to imbalance in DNA methylation, in concert with compromised DNA synthesis and repair processes [33]. There are discrepancies, however, within the literature. Animal based studies propose that insufficiency of folate might inhibit colorectal carcinogenesis, and excessive folate status may promote colorectal carcinogenesis [31,34,35]. Although human epidemiological studies tend to show an inverse relationship between folate status and the risk of developing colorectal cancer there are still concerns that an excessive folate intake may stimulate growth of initiated cancers [36,37]. These observations in epidemiological studies merely propose the association and do not establish a cause and effect relationship between folate intake levels and the risk of colorectal cancer [31]. Folate may have a dual and contrasting effect on cancer, by preventing cancer-initiating chromosomal and epigenetic instability events and potentially stimulating the progression and growth of cancer after initiation (as reviewed in [38]). It is also becoming evident that genetic variation can have a significant bearing on folate transport and metabolism and the interaction of folate intake with genetic variation in folate transport and metabolism genes can influence the observed outcomes of experimental and molecular epidemiological studies of cancer and other diseases [34].

Meanwhile, the scarce data on the relationship between methionine and colon cancer, suggest that methionine may affect the development of colon cancer [6,36]. Methionine restriction inhibits the development of colonic tumours in the colon of rats treated with azoxymethane (AOM), mainly in post-initiation stage of carcinogenesis [37], whilst methionine addition was found to increase the surface area of tumours in the small intestine in the $APC^{Min/+}$ mouse [39].

1.2. Literature search and inclusion criteria

Herein we discuss the results of a thorough review of the current literature on this topic, specifically including only those studies that met the following criteria: (1) use of $APC^{Min/+}$ mouse as the experimental model (this genotype has been notated as $Apc^{+/-}$, Apc^{Min}, and $Apc^{+/Min}$ in other literature); (2) interventions using dietary folate and/or methionine; and (3) primary outcome measures focused specifically on tumour development. Studies that also investigated effects on DNA methylation and genome stability parameters were also considered as this information is relevant for understanding mechanism of action. All references were accessed through publicly available electronic databases, and evaluated by three of the authors (AHT, CB, and MF).

The search used the following electronic databases: Medline, PubMed, Scopus, Web of Science. The reference lists for all obtained studies were hand-searched for additional studies. All potential studies identified were independently evaluated for inclusion by two primary reviewers (AHT and MF). The primary reviewers were not blinded to the authors, institutions or source of publication at any time during the selection process. Disagreements about the inclusion/exclusion of studies were discussed and consensus achieved. Provision was made for a third reviewer if consensus was unattainable but did not prove necessary.

2. The $Apc^{Min/+}$ mouse model of intestinal cancer

The $Apc^{Min/+}$ mouse is an important model for investigating intestinal cancer due to the presence of a genetic mutation in the tumour suppressor gene, adenomatous polyposis coli (Apc). This gene mutation is homologous to human APC germ-line mutations which cause the hereditary condition known as familial adenomatous polyposis (FAP) [40]. An individual with FAP has an elevated risk of developing adenomatous colon polyps [41]. The number of adenomas formed can range from hundreds to thousands, many of which are anticipated to develop into colorectal cancer if left untreated [42]. FAP, attenuated FAP (where there are less than 100 polyps in the colon), and Gardner's syndrome (FAP with osteomas of the skull, epidermoid cysts, dental anomalies, and/or desmoid tumours) all result from germline mutations in the APC gene [43]. APC mutations have also been shown to contribute to the initiation of a majority of sporadic colorectal cancers [44–46], with inactivated APC found in 80% of sporadic colorectal cancer cases [47].

$Apc^{Min/+}$ mice on the C57BL/6 background display multiple intestinal neoplasia (Min) due to the mutant allele encoding a heterozygous mutation at codon 850 of the APC gene [48,49]. As this mutation is very similar to that which causes FAP in humans it represents an ideal model to examine intestinal cancer development, as well as to test potential therapeutics. Similar to humans with APC gene mutation, $Apc^{Min/+}$ mice are predisposed to intestinal adenomas, developing several small intestinal adenomas and a few colorectal adenomas by age 60–120 days of age [49]. Whilst humans with FAP are most likely to develop tumours in the colon, 20–100% of FAP patients also develop tumours in the small intestine (as reviewed in [50]). Unlike humans, however, tumours in the mouse model are more likely to occur in the small intestine [51].

APC is a large multidomain protein, consisting of 2843 amino acids, with several cellular roles including regulation of the WNT signal transduction pathway, cellular adhesion, migration, mitosis and apoptosis [52]. In the WNT signalling pathway (Fig. 2), the APC protein serves as a negative regulator of intracellular levels of β-catenin, by promoting destruction of the latter [53]. In the absence of WNT signal (Fig. 2a), β-catenin is degraded through its interactions with protein Axin, APC, and glycogen synthase kinase 3 (GSK3). In the presence of WNT signal (Fig. 2b), the WNT proteins bind to a transmembrane protein (LRP) receptor. Thus, a signal is transduced to dishevelled (DSH) and Axin, leading to inhibition of the β-catenin degradation [54]. Stabilised β-catenin can then accumulate and enters the nucleus where it interacts with T-cell transcription factor (TCF), forming a heterodimeric transcriptional factor complex, which promotes the transcription of WNT-responsive genes such as c-MYC, cyclin D2 and CD44 leading to cellular hyperproliferation [55,56]. Excessive nuclear accumulation of β-catenin, can lead to developmental abnormalities and tumourigenesis [45].

3. Studies meeting the inclusion criteria

A comprehensive search of published literature generated thirteen studies which met the inclusion criteria of using the $Apc^{Min/+}$ model as the experimental system to investigate the

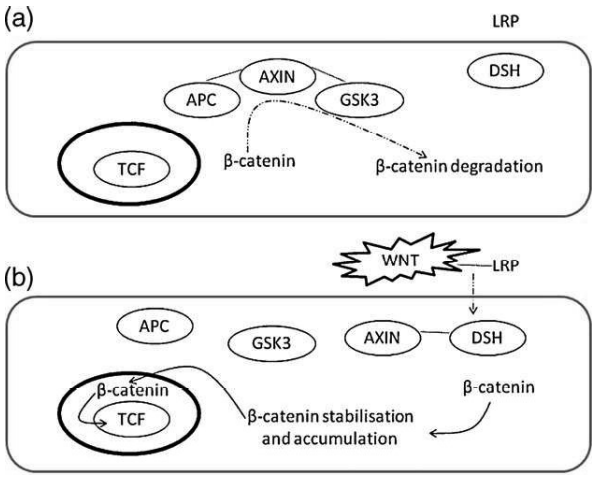

Fig. 2. APC and WNT signalling pathway. (a) In the absence of WNT signal, β-catenin is degraded through its interactions with the proteins Axin, APC, and GSK3; (b) in the presence of WNT signal, the WNT proteins bind to LRP. Thus, a signal is transduced to DSH and Axin, leading to inhibition of the β-catenin degradation. Stabilised β-catenin can then accumulate and enters the nucleus where it interacts with T-cell transcription factor (TCF) causing stimulation of cell division. *Abbreviations*: LRP, LDL receptor related protein; DSH, dishevelled; APC, adenomatous polyposis coli; GSK 3, glycogen synthase kinase 3; TCF, T-cell transcription factor.
Adapted from [54].

effects of dietary folate and methionine on intestinal tumour incidence. A summary of the 13 studies [39,57–68] is presented in Table 1, with highlights of the significant outcomes from each. Fig. 3 provides a diagrammatic representation of the various dietary intervention designs used in these studies. For the purpose of this review and based on the reviewed papers basal adequate requirements of folate and methionine for rodents were considered to be 2 mg of folate and 4.6 g of methionine/kg diet respectively [69,70]. Lesser content was considered deficient, and greater content considered as excess. Deficient diets were either 0 mg folate/kg diet or 0.2–0.4 mg/kg diet with sulphathiazole to suppress growth of folate-producing bacteria in the gut. Diets containing 8 mg or 20 mg folate/kg diet were considered to be excessive for this vitamin.

3.1. Studies which investigated folate deficiency only

Seven studies investigated the effects of folate deficient diet (0 mg/kg diet) relative to folate adequate diet (2 mg/kg diet) in different scenarios of diet and other genetic factors.

Sibani et al. investigated the effect of folate/choline deficiency, together with levels of SAH and SAM, and global DNA methylation in the small intestine in the $Apc^{Min/+}$ mice [60]. Forty-three mice (6–10 per group) were fed a diet deficient for both folate and choline (0 g of folate/kg diet), or a control diet containing 2 mg folate/kg diet at weaning. Mice were killed at 13 weeks of age. Positive linear correlations between SAH or SAM and number of tumours ($p < 0.005$), and between global DNA hypomethylation and tumour multiplicity ($p = 0.014$), were observed in mice receiving the diet containing folate [60]. From their results these researchers concluded that folate/choline deficiency did not produce a consistent effect on tumour number in replicate experiments, possibly because of genetic differences between mice in the different experiments with regards to the transformation state of cells in the intestine and susceptibility to DNA hypomethylation.

Genomic methylation is mediated by DNA (cytosine-5)-methyltransferase 1 (DNMT1) [71]. Trasler et al. studied the effects of reduced DNMT1 expression together with adequate or low dietary folate, to explore the combination of genetic and dietary factors on global DNA methylation and small intestinal tumour development [61]. Promoter CpG island methylation profile of E-cadherin and p53 genes were also evaluated. To achieve the objectives, the $DNMT^{(+/-)}$ mutant $Apc^{Min/+}$ mouse model was used. Two experiments were carried out with 77 mice ($n = 29$ for experiment 1, and $n = 48$ for experiment 2, with $n = 3$–14 per group) given either a folate deficient diet (0 mg of folate/kg diet) or a control diet (2 mg folate/kg diet) post weaning. Mice were sacrificed at 13 weeks. With regard to DNMT1, $Apc^{Min/+}$ mice with mutant DNMT1 had significantly fewer tumour numbers compared to $Apc^{Min/+}$ with normal DNMT1, in both the folate deficient and control group. Assessment of the dietary and genetic manipulation showed that folate deficiency in the $Apc^{Min/+}$ mice with mutant DNMT1 resulted in decreased tumour numbers, compared to other groups. However, the hypothesis that low level of dietary folate could expedite tumorigenesis in the $Apc^{Min/+}$ was

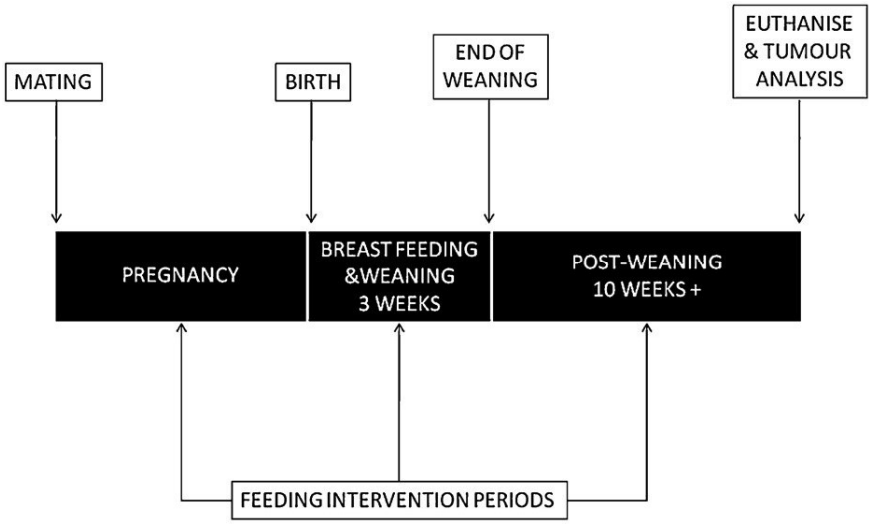

Fig. 3. Summary of typical dietary intervention study designs used for folate and/or methionine interventions in $Apc^{Min/+}$ mouse studies. Intervention studies varied depending on when the dietary change intervention was commenced or finalized. The intervention periods investigated were either (i) during pregnancy, (ii) for the first 3 weeks after birth when baby mice are still being breast fed and/or weaned and (iii) post-weaning. Typically mice were sacrificed 10 weeks or later after weaning. Most of the studies performed interventions in the post-weaning period.

Table 1
Summary of published studies using the $Apc^{Min/+}$ mouse model to examine the effect of folate and/or methionine on tumour development and genome stability in chronological order of publication.

Reference and description	Genotype (gender)	Sample size (*n* per group)	Dietary intervention (base diet)	Start time of intervention	Duration of intervention (weeks)	Important outcomes
Song et al. [57] Investigate effects of folate	$Apc^{Min(+/-)}$ (male and female)	79 (8–11)	0, 2.0, 8.0, 20.0 mg folate/kg diet (amino acid-defined diet)	Post weaning (3 weeks of age)	12–13, 25–26	Increasing folate decreased number of ileal polyps. Increasing folate decreased number of colonic aberrant crypt foci
Song et al. [58] Investigate effects of folate	$Apc^{Min(+/-)}Msh2^{(-/-)}$ (male and female)	31 (7–10)	0, 8.0 mg folate/kg diet (amino acid-defined diet)	Post weaning (3 weeks, i.e. before establishment of neoplastic foci or 6 weeks of age, i.e. after establishment of neoplastic foci)	5, 8	High folate decreased number of adenomas in small intestine Folate supplementation from 3 weeks reduced intestinal adenomas, colonic ACF, and colonic adenomas by 2.7-2.8 fold Folate supplementation from 6 weeks reduced small intestinal adenomas by 4.2 fold
Paulsen and Alexander [39] Investigate methionine effects	$Apc^{Min(+/-)}$ (male and female)	16 (3–5)	4.6 g, 11.6 g methionine/kg diet (AIN-76)	Post weaning (4 weeks of age)	4	Number of tumours unchanged between treatments Excess methionine increased surface area of small intestinal tumours
Tucker et al. [59] Investigate effects of 5-fluorouracil and folate	$Apc^{Min(+/-)}$ (male and female)	274 (6–37)	0, 4.0, 6.0 mg folic acid/kg diet (standard rodent chow)	Post weaning (4 weeks of age)	7, 12	Increasing folate increased tumour number
Sibani et al., 2002 [60] Investigate SAM, SAH, DNA methylation and folate/choline deficiency effects	$Apc^{Min(+/-)}$ (male and female)	43 (6–10)	0, 2.0 mg folate/kg diet (AIN-like, semi-synthetic diet)	Post weaning (3 weeks of age)	10	Folate/choline did not produce a consistent effect on tumour number Folate/choline deficiency decreased SAM Folate/choline deficiency increased global DNA hypomethylation Positive correlation between SAM, SAH or DNA methylation with tumour multiplicity
Trasler et al. [61] Investigate DNMT1 and folate deficiency effects	$Apc^{Min(+/-)}DNMT1^{(+/-)}$ (male and female)	77 (3–14)	0, 2.0 mg folate/kg diet (AIN-like semi-synthetic diet)	Post weaning (3 weeks of age)	10	Folate deficiency decreased tumour number in $Apc^{Min(+/-)}DNMT1^{(+/-)}$, but not in $Apc^{Min(+/-)}DNMT1^{(+/+)}$ mice DNMT1 deficiency with or without folate deficiency decreases tumour number in $Apc^{Min(+/-)}$ mice
Bashir et al. [62] Investigate folate in the context of multiple vitamin deficiency or excess	$Apc^{Min(+/-)}$ (female)	180 (30)	0.3, 1.0, 2.0 mg folate/IU semi synthetic diet with altered vitamin content	Post weaning (3 weeks of age)	8	Reduced or increased folate conditions in parallel with corresponding multivitamin changes increased number of polyps and the tumour burden, crypt fission and weight of small intestine
Lawrance et al. [63] Investigate interaction effect of Rfc1 knockout, MTR knockout and folate deficiency effects	$Apc^{Min(+/-)}Rfc1^{(+/-)}$, $Apc^{Min(+/-)}MTR^{(+/-)}$ (male and female)	79 (3–16)	0.2-0.3, 2.0 mg folic acid/kg diet (AIN-93)	Post weaning (3 weeks of age)	7	Folate deficiency increased adenoma number, plasma homocysteine, apoptosis in all genetic backgrounds Folate deficiency decreased global DNA methylation in all genetic backgrounds Rfc1 deficiency reduced adenoma number and load, but MTR deficiency had no such effect
McKay et al. [64] Investigate *in utero* folate deficiency effects	$Apc^{Min(+/-)}$ (male and female)	148 (37)	0.26, 0.4, 2.0 mg folic acid/kg diet (AIN-93G)	*In utero* and after birth (intervention starts on breeding mice, throughout pregnancy and nursing)	10	No significant effect of folate dose on tumour number or size

Lawrance et al. [65] Investigate MTHFR and folate effects	$Apc^{Min(+/-)}MTHFR^{(+/-)}$ (male and female)	59 (8–13) 70 (2–12)	0.3, 2.0, 20.0 mg folate/kg diet 0.3, 2.0, 20.0 mg folate/kg diet (amino acid-defined diet)	Post weaning (3 weeks of age) offspring (dietary intervention started prior to mating, throughout pregnancy and nursing)	7+	High folate diet from weaning developed more adenomas MTHFR had no impact Offspring of mice on low folate diet and subsequently on the same diet post-weaning developed fewer adenomas $MTHFR^{+/-}$ genotype associated with reduced adenoma numbers in the $Apc^{Min/+}$ offspring
MacFarlane et al. [66] Investigate Mthdf1 and folate deficient effects	$Apc^{Min(+/-)}Mthdf1^{(+/-)}$ (male and female)	32 (8)	0, 2.0 mg folate/kg diet AIN-93G	Post weaning (3 weeks of age)	11	Folate deficiency and Mthdf1 had no effect on tumour in the $Apc^{Min/+}$ mice
MacFarlane et al. [67] Investigate SHMT1 and folate effects	$Apc^{Min(+/-)}SHMT1^{(+/-)}$ (male and female)	NA (4–13)	0, 2.0 mg folate/kg diet AIN-93G	Post weaning (3 weeks of age)	11	Folate deficiency increased tumor number and load in $Apc^{Min/+}SHMT1^{-/+}$, but had no effect in $Apc^{Min/+}SHMT1^{+/+}$, or $SHMT1^{-/-}$ mice Folate deficiency increased uracil content in $Apc^{Min/+}$ mice with SHMT1 knockout
McKay et al. [68] Investigate maternal folate deficiency effects on DNA methylation in the offspring	$Apc^{Min(+/-)}$ (male and female)	NA (10–21)	0.4, 2.0 mg folate/kg diet AIN-93G	*In utero* and after birth (intervention starts on breeding mice, throughout pregnancy and nursing)	9+	Maternal folate deficiency reduced p53 methylation in adult mice Folate deficiency post-weaning increased APC methylation in the $Apc^{Min/+}$ mice

Abbreviations: SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; DNMT1, DNA (cytosine-5)-methyltransferase 1; Rfc1, reduced folate carrier 1; MTHFR, methylenetetrahydrofolate reductase; Mthdf1, methylenetetrahydrofolate dehydrogenase; SHMT1, cytoplasmic serine hydroxymethyltransferase.

not proved. A 10-week (from weaning to 13 weeks of age) period of folate deficiency did not accelerate tumorigenesis in $Apc^{Min/+}$ mice (without DNMT1 mutation). Folate deficiency in this study resulted in decreased concentration of SAM, which is essential for DNA methylation. However, none of the mice in the folate deficient diet group showed changes in DNA methylation. In summary this study showed that folate deficiency was marginally associated with decreased tumour number, and did not accelerate tumorigenesis. Folate intake did not influence overall genomic DNA methylation or the methylation levels of E-cadherin or p53 in intestinal tissue, regardless of the presence or absence of functional DNMT1 [61].

A study by Lawrance et al. used a genetically modified $Apc^{Min/+}$ mouse, which was genetically deficient in cellular folate uptake or folate metabolism, to study the effects of folate deficiency on tumorigenesis [63]. To generate these double mutant strains the $Apc^{Min/+}$ mice were crossed with reduced folate carrier 1 (Rfc1) knockout mice or with methionine synthase (MTR) knockout mice. Rfc1 acts as transporter of 5-methylTHF into cells whereas MTR is the enzyme required for transferring the methyl group from 5-methylTHF to homocysteine to produce methionine, which is then converted to SAM. Seventy-nine mice (3–16 per group) were assigned to receive either a control diet containing 2 mg folic acid/kg or a folic acid deficient diet containing 0.2–0.3 mg folic acid/kg. Mice were killed at 10 weeks of age. Results showed that folate deficiency increased the number of adenomas in both $Mtr^{+/+}Apc^{Min/+}$ and $Mtr^{+/-}Apc^{Min/+}$ mice, although dietary folate appeared to have no significant influence on size of adenomas. The folate deficient diet caused a significant increase in plasma homocysteine in all genotypes ($Mtr^{+/+}Apc^{min/+}$, $Mtr^{+/-}Apc^{Min/+}$, $Rfc1^{+/+}Apc^{Min/+}$ and $Rfc1^{+/-}Apc^{Min/+}$), as well as an increase in caspase-3/7 activity suggesting an increased rate of apoptosis. It was also found that the $Apc^{Min/+}$ mice with knockout Rfc1 developed fewer adenomas, as well as reduced adenoma sizes [63]. From this study, it was concluded that genetic and nutritional alterations in folate bioavailability may impact tumorigenesis in $Apc^{Min/+}$ mice.

A study by McKay et al. hypothesised that insufficient folate *in utero* and during early life may affect tumorigenesis in the offspring [64]. Female C57Bl6/J mice were randomised to a folate adequate (2 mg folic acid/kg diet) or folate deficient (0.4 mg folic acid/kg) diet throughout mating (with $Apc^{Min/+}$ male), pregnancy and lactation. 148 $Apc^{Min/+}$ offspring (37 per group) were then randomly assigned to receive folate adequate (2 mg folic acid/kg diet) or folate deficient diet (0.26 mg folic acid/kg) for 10 weeks post-weaning. Results showed no evidence that maternal folate deficiency affected tumorigenesis in the offspring. However, female offspring mice fed normal folic acid diet post-weaning had more and larger tumours compared to folate-depleted females post-weaning.

Two recent studies by MacFarlane et al. [66,67], used genetic modification of methylenetetrahydrofolate dehydrogenase 1 (Mthfd1) and cytoplasmic serine hydroxymethyltransferase (SHMT1) to study effects of folate deficiency in the $Apc^{Min/+}$ mice. Both Mthfd1 and SHMT1 play an important role in folate metabolism, with Mthdf1 providing folate-activated-one-carbon units in the cytoplasm [66], whilst SHMT1 is required for the synthesis of 5,10-methylenetetrahydrofolate which is the form of folate essential for synthesis of dTTP from dUMP [67]. The first study [66] compared two models of intestinal cancer; (i) crosses between $Mthfd1^{gt/+}$ and $Apc^{Min/+}$ mice, and (ii) AOM (chemically)-induced colon cancer in $Mthfd1^{gt/+}$ mice. Post-weaning, mice were grouped in $n = 8$ per group, and randomly assigned onto control diet containing 2 mg folic acid/kg diet or folate deficient diet containing 0 mg folic acid/kg diet. Dietary interventions were maintained for 11 weeks. *Mthfd1* had no significant effect on

tumour incidence in the $Apc^{Min/+}$ mice. However, a non-significant trend indicating a reduction in tumour number and size was recorded in groups fed the folate deficient diet which caused a decrease in folate concentration compared to the control group.

The second recent study by this group [67] looked at the interaction of SHMT1 gene and dietary folate in modifying susceptibility to intestinal cancer in the $Apc^{Min/+}$ mice. Mice were fed with either a folate adequate control diet with 2 mg folic acid/kg diet or folate deficient diet containing 0 mg folic acid/kg diet. Mice were maintained on the dietary regimen from 3 to 14 weeks of age. Folate deficiency decreased tissue folate concentration, increased total tumour number and load in $Apc^{Min/+}SHMT1^{-/+}$, but had no effect in $Apc^{Min/+}SHMT1^{+/+}$, or $SHMT1^{-/-}$ mice. It was also found that folate deficiency increased uracil content in hepatic nuclear DNA in $Apc^{Min/+}$ mice with SHMT1 knockout.

Recently, McKay et al. [68] published their investigation on effects of maternal folate deficiency on DNA methylation in the offspring. Female C57Bl6/J mice were given either a folate replete (2 mg folic acid/kg diet) or low folate (0.4 mg folic acid/kg diet) diet, from mating with $Apc^{Min/+}$ males, throughout pregnancy and lactation. Offspring (both $Apc^{Min/+}$ and wild type) were randomly assigned to receive low or high folate diets, post-weaning to an average of 96 days of age. Methylation of specific genes (pf3, Igf2, p16 and APC) were assessed at weaning and in the adult offspring. Overall, it was found that maternal folate depletion reduced p53 methylation in adult offspring. Folate deficiency post-weaning increased APC methylation in the $Apc^{Min/+}$ mice only.

3.2. Studies with both folate deficiency and excess

Four out of 13 studies were done with both deficient and excess levels of dietary folate and effects assessed relative to adequate levels of folate in the diet (i.e. 2 mg/kg diet; excess levels were typically 8 mg folate/kg diet or greater). The first by Song et al. was designed to investigate the effects of 0, 2, 8 and 20 mg/kg of folate in the diet on the development of intestinal polyps in $Apc^{Min/+}$ mice [57]. Seventy-nine mice (8–11 per group) were fed with assigned diets from weaning, prior to sacrifice following either 3 or 6 months on the intervention. Adenomas in the small intestine and aberrant crypt foci (ACF), and adenomas in the colon were determined. Increasing folate was found to cause a significant reduction in the number of ACF in the colon after 3 months. Whilst at 6 months, no significant differences in ACFs were observed amongst all groups. After 3 months of dietary intervention, however, the higher folate diets were shown to significantly reduce the number of adenomas in the ileum, with animals in the 20 mg/kg diet group showing a 68–78% reduction in the number of ileal adenomas compared with the three lower folate diets. After 6 months intervention this effect had reversed, with the group given the diet containing 0 mg of folate/kg displaying lower numbers of ileal adenomas than the other three diets. In summary, results obtained from this study suggest that dietary folate supplementation reduced the development of ACF in the colon in the short term but not in the long term. The results for the development of adenomas in the ileum were conflicting and were dependent on duration of intervention. Serum folate concentration was lowest in the group fed with 0 mg of folate/kg, compared to other groups, at both 3 months and 6 months time point; however, the mean folate concentration within each group was not significantly different at 3 months and 6 months indicating that the changes in adenoma formation with time were not confounded by fluctuations in tissue folate concentration with time.

Another study by the same group was carried out using an $Apc^{Min/+}$ model with a null mutated *Msh2* gene ($Msh2^{-/-}$) designed to investigate the extent to which folate in the diet may affect the progress of developing intestinal tumorigenesis [58]. During cell division, *Msh2*, a mismatch repair gene is involved in maintaining fidelity of genome replication, and defects in this type of DNA repair gene are associated with increased risk of intestinal cancer in human [72]. Mice were randomly assigned to receive a diet containing either 0 or 8 mg of folate/kg starting from weaning at 3 weeks of age (before the development of neoplastic foci), or 3 weeks later (after the development of neoplastic foci). Mice were sacrificed at 11 weeks of age. As with the previous study from this group, ACFs in the colon and adenomas in the small intestine and colon were analysed. Folate supplementation starting at the end of weaning (at 3 weeks) was found to significantly reduce the number of adenomas in both small intestine and colon. In contrast, folate deficient diet, which caused a drop in serum folate concentration, significantly reduced the number of adenomas in the small intestine if commenced after the development of neoplastic foci (6 weeks). However there was no effect on colon ACFs and adenomas. Based on their analyses of global methylation, these researchers concluded that there was no significant relation between genomic DNA methylation and the development of tumorigenesis in this study, regardless of the duration and starting point of folate interventions. In summary the researchers concluded from this work that dietary folate supplementation before neoplastic foci formation attenuates the development of tumorigenesis in both the small intestine and colon. However, timing of the intervention was shown to be important because folate supplementation after development of neoplastic foci (i.e. at 6 weeks of age) resulted in a 4.2 fold increase in incidence of small intestinal adenomas.

Later, a study carried out by Tucker et al. investigated the response of 5-fluorouracil chemotheraphy with dietary folic acid (at 0, 4.0 and 6.0 mg/kg diet) in $Apc^{Min/+}$ model [59]. Mice (n = 6–37 per group) were assigned to received dietary regimens starting at 4 weeks of age, and tumour number were evaluated at 10 and 15 weeks of age. In general, their data suggest that mice on folate deficient or excess diet developed lesser tumour number, compared to mice fed with standard rodent chow diet (containing 2 mg folic acid/kg diet).

A more recent study by Lawrance et al. determined the combined effects of MTHFR genotype and folate deficiency on tumorigenesis in the $Apc^{Min/+}$ genetic background [65]. Diets with variable folate content (0.3, 2.0, 20.0 mg folic acid/kg diet) were administered either pre-natally or at weaning. Mice fed high folate diets from weaning developed more adenomas than those on low folic acid or deficient diets, but MTHFR genotype had no impact. Offspring of dams fed with folate deficient diet and subsequently on the same diet post-weaning developed fewer adenomas; $MTHFR^{+/-}$ genotype of the mother, or of the offspring, was also associated with reduced adenoma numbers in the $Apc^{Min/+}$ offspring. Adenoma number was inversely associated with plasma homocysteine and intestinal dUTP/dTTP ratio [65].

3.3. Study with folate as a multivitamin component

There is a distinctive study conducted by Bashir et al. [62], designed to test the effects of altering the content of multiple vitamins in the $Apc^{Min/+}$ mouse model. One of the vitamin components that was altered in the diet was folate. 180 mice (30 per group) were used to examine the effects of modified vitamin levels on the development of intestinal polyps, cell proliferation and crypt fission (a physiologic mechanism of crypt reproduction). Modifications were made to the concentration of several vitamins and micronutrients in the diet (A, B1, B2, B3, B5, B6, and B12, folate, biotin, C, D, E, K and selenium). At the age of 4 weeks, mice were assigned either to the control diet (basal levels of vitamins and micronutrients, containing 1.0 mg folate/kg diet), low vitamin diet (depleted vitamin and micronutrient content, containing 0.33 mg folate/kg diet), or high vitamin diet (replete

vitamin and micronutrient content, containing 2.0 mg folate/kg diet). Animals were killed at 12 weeks of age, after 8 weeks on the treatment diets. Polyps, cell proliferation and crypt fission were then evaluated. The diets containing both low and high folate resulted in an increase in polyp number in the small intestine, compared to controls. Both low and high folate supplementations (as part of the diet contents) showed a small decrease in cell proliferation. Only the diet containing high folate content had a significant effect on decreasing crypt fission. The authors acknowledged the possible role of folate in providing such effects [62], although interactions with other vitamin intake changes might be involved. This study is unique, and not strictly comparable to other studies mentioned earlier due to a few factors: (1) amount of folate was modified together with other vitamins which makes it impossible attribute the effects solely to folate; and (2) definition of high and low folate differs from other studies, because 2.0 mg of folate is classified as high in this study whereas 2.0 mg of folate is considered as basal dosage in other studies discussed above.

3.4. Study with methionine

To our knowledge only one study investigating the effects of dietary methionine supplementation in the development of intestinal adenomas in the $Apc^{Min/+}$ mouse model has been reported to date, by Paulsen and Alexander [39]. Sixteen mice (3–5 per group, mixed male and female) were assigned either to the standard AIN-76A diet (containing 4.6 g of methionine/1 kg diet) or the AIN-76A diet with added 0.7% L-methionine (equivalent to 11.6 g of methionine/1 kg diet) for 4 weeks, commencing from 4 weeks of age, prior to sacrifice at the age of 8 weeks. Results showed that the additional methionine promoted the growth of adenomas in the small intestine by increasing the surface area of tumours by 41%, whilst having no affect on the number of tumours in both the small intestine and the colon [39]. The very short duration of treatment in this study makes it difficult to compare with folate intervention studies which were all carried out for longer duration.

4. Proposed mechanisms for the effects of folate and methionine on intestinal tumour growth in $Apc^{Min/+}$ mice

The mechanisms by which dietary methionine and/or folate may affect intestinal carcinogenesis are speculative at this stage. There are at least three possibilities: (1) modulation of nucleotide synthesis caused by folate deficiency or excess; (2) DNA methylation changes due to deficiency of methyl donors which may alter gene expression; and (3) altered polyamine metabolism which may affect the rate of cellular division.

4.1. Modulation of nucleotide synthesis caused by folate deficiency or excess

Folate (in the form of formyl THF) is essential for one carbon metabolism and involved in the conversion of dUMP to dTMP, and in the synthesis of purines [73].

Severe folate deficiency leads to an excess of dUMP and increased incorporation of uracil in DNA which is mutagenic and impairs DNA synthesis and repair [74–76]. Folate insufficiency is known to imitate clastogenic genotoxin exposure by inducing single and double strand breakage and/or oxidative lesions [77]. Mechanistically, this effect arises from a reduction in the pool of DNA precursors, transient DNA breaks caused by excision repair of uracil from DNA thus inhibiting proliferation and DNA synthesis in multiplying adenoma cells [63]. As such it is proposed that folate deficiency may slow tumour growth by increasing DNA damage [63] and cell cycle delay by checkpoint mechanisms (as reviewed in [78]). The replication stress caused by impaired folate metabolism inhibits cell division which is the central principle of methotrexate and 5-fluorouracil, both anti-folate agents, used as antitumour therapies [79].

In the $Apc^{Min/+}$ mouse model the data is conflicting, with some studies showing folate having an inhibitory effect, whilst in other circumstances it is stimulatory of tumorigenesis. The effects appear to be dependent on the timing and dosage of the intervention. For example, it has been suggested that dietary folate may suppress the development of polyps when implemented at an early stage (upon weaning), but may show an opposite effect when given at later stage [57,80]. Genetic defects which alter folate transport proteins such as reduced folate carrier (Rfc1) [81], could also affect the formation of adenoma in this model [61,63]. In contrast, in mouse models which were genetically normal for APC but had folate binding protein (Folbp1) or Rfc1 genes ablated, treatment with the colon carcinogen azoxymethane resulted in larger adenocarcinomas in Rfc1 deficient mice and more tumours occurred in the Folbp1 defective mice relative to their genetically normal counterparts [73]. These conflicting results suggest that folate might have opposing effects acting as an inhibitor at the initiation phase of carcinogenesis which is driven by mutagenesis, whilst acting as promoter in the latter stages by stimulating cellular proliferation [62]. However, because tumours may be initiated or promoted at different time-frames along the intestine, there is likely to be some overlap in their chronicity which creates uncertainties for determining the appropriate timing for folate interventions.

In the murine model, weaning offspring are dependent on maternal supply of folate (in the form of 5-MeTHF) from their mother's milk, and their own folate uptake mechanisms [51,63,82]. It has been shown that intestinal cells are more prone to folate-deficiency induced adenoma formation in the newborn, compared to adult mice [51]. This supports the significance of folate as an inhibitor at the initiation phase of carcinogenesis.

4.2. DNA methylation changes due to deficiency of methyl donors which may alter gene expression

One of the speculated mechanisms underlying the effect of dietary folate and/or methionine on intestinal carcinogenesis is through alterations in DNA methylation. Deficiency in these micronutrients results in reduced levels of SAM, altered DNA methylation patterns and enhanced mutation rates [83]. DNA methylation is inversely linked with gene expression, with methylation of CpG islands adjacent to promoters down-regulating gene transcription [84,85]. Alterations in DNA methylation and levels of DNA methyltransferase expression have been associated with tumorigenesis [86,87]; specifically global DNA hypomethylation has been reported in colonic neoplasia [88,89]. Methyl group deficient diets lead to DNA hypomethylation in rats [90–92], and in humans [93]. In pre-neoplastic small intestinal cells, global DNA hypomethylation is positively correlated with tumour multiplicity in mice fed adequate folate [60]. These findings are in agreement with a previous study showing DNA hypomethylation in neoplastic cells (from human colon tissue) [94,95]. Interestingly, Trasler et al. demonstrated that folate deficiency can decrease tumour number without influencing overall genomic DNA methylation status, suggesting that other mechanisms (e.g. uracil incorporation into DNA) are also implicated in tumorigenesis [61]. On the other hand, aberrant DNA methylation also affects the development of mutations by causing incorrect transcriptional control of tumour suppressor (e.g. silencing of DNA repair and cell cycle checkpoint genes) and tumour promoter genes (e.g. activation of parasitic oncogenic viral DNA) consequently increasing tumour risk [96].

4.3. Altered polyamine metabolism which may affect the rate of cellular division

Intestinal epithelium may utilise methionine generated in the folate/methionine metabolic cycle (Fig. 1) or dietary methionine to meet the requirement of polyamines for cellular hyperproliferation in tumorigenesis [16,97]. There is an inter-related connection between polyamine synthesis, WNT signalling and the APC tumour suppressor gene in intestinal epithelium. APC down-regulates β-catenin. WNT signals modulate the stability of a protein complex (containing β-catenin). In the absence of WNT or the presence of wild-type APC protein, β-catenin is degraded by the 26 S proteasome. In contrast, in the presence of WNT, or the absence of APC, β-catenin target genes (including c-*myc*) are expressed. The expression of c-myc leads to the increased expression of ornithine decarboxylase (ODC), an enzyme involved in polyamine synthesis which leads to polyamine-induced increase in cell proliferation [98]. The synthesis of polyamines is ultimately dependent on methionine supply because decarboxylated S-adenosyl methionine is the precursor molecule essential for polyamine synthesis [58]. Folate and methionine status possibly modulate the initiation of tumorigenesis through their role in polyamine production [97,98]. Hence, the higher SAM observed in the pre-neoplastic small intestine of $Apc^{Min/+}$ mice may signify an increased potential for polyamine synthesis [60]. In contrast polyamine depletion was shown to reduce adenoma development in the $Apc^{Min/+}$ mouse model [99].

5. Knowledge gaps and conclusions

With the limited literature available, the mechanisms by which dietary methionine or folate may affect tumorigenesis in the $Apc^{Min/+}$ mouse model remain unclear and speculative. Although this model has been used extensively in investigating dietary effects of intestinal cancer the mice mainly develop small intestinal polyps in contrast to humans for whom intestinal cancer develops mainly in the colon [57]. In this review, we focussed on the role of methionine and folate, although it should be noted that there are other components of the mouse diets that may vary between studies such as protein, fat, carbohydrate and vitamin contents which could further modify susceptibility to carcinogenesis. As a result, several key questions still need to be addressed to clarify our understanding of the beneficial or detrimental effects of dietary folate and methionine on intestinal tumorigenesis in $Apc^{Min/+}$ mouse model such as:

- What are the optimum intakes of folate and methionine in ageing mice to prevent growth of intestinal cancer?
- When is the right time to implement folate and methionine restriction or supplementation to prevent or control cancer growth?
- How do different genetic and dietary backgrounds alter the effects of folate and methionine deficiency or excess on the conversion of a benign polyp to an adenoma?
- Is there an interactive effect of folate and methionine and which of the two has the strongest impact on intestinal tumour incidence and progression?
- What are the gene expression pattern changes that diagnose a trajectory to malignancy in this model?

Sex-specific effects of folate and/or methionine on tumour development, was rarely tested or discussed in the reviewed studies. Only one study [64] discussed this, and it was found that folate deficiency post weaning has a protective effect against neoplasia in female $Apc^{Min/+}$ mice, but not in male mice. Whether sex is a strong modifying genetic factor remains unresolved and

Table 2
Mean small intestinal tumour frequency per mouse reported in published studies for $Apc^{Min/+}$ mice fed either folate-deficient, -adequate or -excessive diets during the post-weaning phase (i.e. after the age of 21 days).

Publication	Genotype	Age at diet start	Age at sacrifice	Number of SI tumours per mouse by folate diet		
				Deficient	Adequate	Excessive
Song et al. [57]	$Apc^{Min(+/-)}$	21 days	12 weeks	24	23	19
	$Apc^{Min(+/-)}$	21 days	24 weeks	18	26	21
Song et al. [58]	$Apc^{Min(+/-)}$, $Msh^{(2-/-)}$	21 days	11 weeks	229	ND	110
Sibani et al. [60]	$Apc^{Min(+/-)}$	21 days[a]	13 weeks	16	36	ND
	$Apc^{Min(+/-)}$	21 days[a]	13 weeks	35	24	ND
	$Apc^{Min(+/-)}$	21 days[a]	13 weeks	35	19	ND
Trasler et al. [61]	$Apc^{Min(+/-)}$, $DNMT1^{(+/+)}$	21 days	13 weeks	53	61	ND
	$Apc^{Min(+/-)}$, $DNMT1^{(c/+)}$	21 days	13 weeks	22	20	ND
	$Apc^{Min(+/-)}$, $DNMT1^{(c/+)}$	21 days	13 weeks	26	40	ND
Lawrance et al. [63]	$Apc^{Min(+/-)}$, $Rfc1^{(+/+)}$	21 days	10 weeks	57	60	ND
	$Apc^{Min(+/-)}$, $MTR^{(+/+)}$	21 days	10 weeks	50	33	ND
	$Apc^{Min(+/-)}$, $Rfc1^{(+/-)}$	21 days	10 weeks	42	30	ND
	$Apc^{Min(+/-)}$, $MTR^{(+/-)}$	21 days	10 weeks	43	30	ND
McKay et al. [64]	$Apc^{Min(+/-)b}$	21 days	13 weeks	8	12	ND
	$Apc^{Min(+/-)c}$	21 days	13 weeks	9	14	ND
Lawrance et al. [65]	$Apc^{Min(+/-)}$, $MTHFR^{(+/+)}$	21 days	10 weeks	22	29	41
	$ApcMin^{(+/-)}$, $MTHFR^{(+/-)}$	21 days	10 weeks	25	25	38
MacFarlane et al. [66]	$Apc^{Min(+/-)}$	21 days[a]	14 weeks	24	29	ND
	$Apc^{Min(+/-)}$, $Mthfd1^{(gt/+)}$	21 days[a]	14 weeks	21	21	ND
MacFarlane et al. [67]	$Apc^{Min(+/-)}$, $SHMT^{(+/+)}$	21 days[a]	14 weeks	39	40	ND
	$Apc^{Min(+/-)}$, $SHMT^{(-/+)}$	21 days[a]	14 weeks	59	31	ND
	$Apc^{Min(+/-)}$, $SHMT^{(-/-)}$	21 days[a]	14 weeks	33	48	ND

Notes: Deficient = 0 mg/kg or 0.2–0.4 mg/kg with sulphathiazole; adequate = 2 mg/kg; excessive = 8 mg/kg or 20 mg/kg. ND = not done.
[a] Folate deficient diets were also deficient in choline.
[b] Maternal diet was folate deficient.
[c] Maternal diet was folate adequate.

Table 3
Mean colon tumour frequency per mouse reported in published studies for $Apc^{Min/+}$ mice fed either folate-deficient, -adequate or -excessive diets during the post-weaning phase (i.e. after the age of 21 days).

Publication	Genotype	Age at diet start	Age at sacrifice	Number of tumours in colon per mouse by folate diet		
				Deficient	Adequate	Excessive
Song et al. [57]	$Apc^{Min(+/-)}$	21 days	12 weeks	4.6	4.2	3.7
	$Apc^{Min(+/-)}$	21 days	24 weeks	2.6	2.8	3.2
Song et al. [58]	$Apc^{Min(+/-)}$, $Msh^{(2-/-)}$	21 days	11 weeks	1.7	ND	0.6
McKay et al. [64]	$Apc^{Min(+/-)}$b	21 days	13 weeks	0.7	0.3	ND
	$Apc^{Min(+/-)}$c	21 days	13 weeks	0.4	0.3	ND
MacFarlane et al. [66]	$Apc^{Min(+/-)}$	21 days[a]	14 weeks	0.6	1.6	ND
	$Apc^{Min(+/-)}$, $Mthfd1^{(gt/+)}$	21 days[a]	14 weeks	0.7	0.7	
MacFarlane et al. [67]	$Apc^{Min(+/-)}$, $SHMT^{(+/+)}$	21 days[a]	14 weeks	0.6	1.3	ND
	$Apc^{Min(+/-)}$, $SHMT^{(-/+)}$	21 days[a]	14 weeks	2	1	ND
	$Apc^{Min(+/-)}$, $SHMT^{(-/-)}$	21 days[a]	14 weeks	1.2	1.1	ND

Notes: Deficient = 0 mg/kg or 0.2–0.4 mg/kg with sulphathiazole; adequate = 2 mg/kg; excessive = 8 mg/kg or 20 mg/kg. ND = not done.
[a] Folate deficient diets were also deficient in choline.
[b] Maternal diet was folate deficient.
[c] Maternal diet was folate adequate.

needs to be investigated with adequately powered studies. To date the majority of the studies have investigated the relative effect of folate deficient and folate adequate diets on small intestinal tumours in the $Apc^{Min/+}$ mouse including such mice with defects in one-carbon or folate–methionine metabolism genes. We collated together the reported numerical data for small intestinal and colon tumour frequency per mouse from these studies (Tables 2 and 3) and all together it is evident that there is no significant effect of folate intake on tumour number in the $Apc^{Min/+}$ model (Fig. 4). The

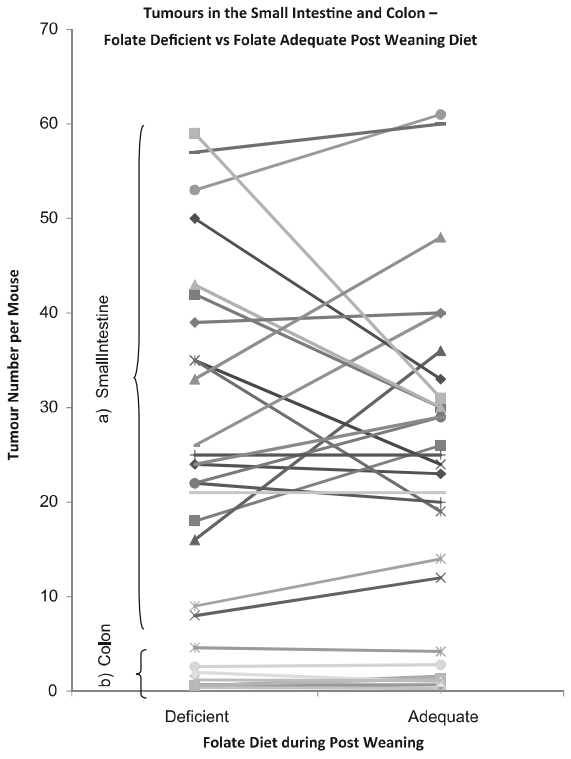

Fig. 4. (a) Small intestinal tumour frequency per mouse in $Apc^{Min/+}$ mice that were fed folate deficient (0–0.4 mg/kg) or folate adequate diets (2 mg/kg) post-weaning in experiments reported in eight published studies [57,58,60,61,63–67]. Some studies reported more than one experiment. For further details refer to Table 2. (b) Colon tumour frequency per mouse in $Apc^{Min/+}$ mice that were fed folate deficient (0–0.4 mg/kg) or folate adequate diets (2 mg/kg) post-weaning in experiments reported in four published studies [57,58,64–67]. Some studies reported more than one experiment. For further details refer to Table 3.

limited data on the effect of folate excess also suggest a lack of impact on tumour incidence when data are pooled together (Tables 2 and 3). This therefore raises the concern that the results of single small studies may be unreliable on their own and in fact inconsistencies in repeat experiments within the same laboratory using the same $Apc^{Min/+}$ model have been reported [55].

Whether the results of experiments in $APC^{Min/+}$ mice on folate and methionine may reflect the effects on intestinal cancer in wild type or genetically normal mice is unclear. However, a study by Newmark et al. [100] showed increased intestinal neoplasms in normal C57/BL6 mice that were fed a "western-style diet" that was reduced in calcium, vitamin D, folic acid, methionine, choline and vitamin B12 to an extent approximating that consumed by western human populations. Subsequent studies by the same group using this model showed that the "western-style diet" induced changes in gene expression in colonic epithelium similar to that caused by inheritance of a mutant APC gene [101]. Furthermore, these studies showed that supplementation of the "western style diet" with calcium and vitamin D suppressed colon tumours but there was no benefit following supplementation with folic acid, methionine, choline and vitamin B12 [102].

The conflicting data from individual small studies on the effect of folate on tumorigenesis requires further investigation with statistically more robust designs, including measurements of folate derivatives and other relevant indicators (such as expression of folate and methionine receptors). The scarcity of data on the (possibly harmful) effects of excess dietary methionine in APC defective genetic backgrounds emphasises the need for further investigation and whether this is influenced by the methionine-dependence phenotype resulting from deletion of methylthioadenosine phosphorylase (MTAP) gene required for regeneration of methionine in cells [103]. Furthermore, more attention should be given to other mechanisms by which folate and methionine affect gene expression; for example folate deficiency or excess can alter histone methylation and microRNA profiles but the extent to which these factors play a role in intestinal carcinogenesis in $APC^{Min/+}$ mice remains unclear [104,105]. Therefore, more folate and methionine-dependent tumorigenesis mechanisms should be studied, such as changes in histone and DNA methylation status, assessment of chromosomal stability and DNA damage, the influence of SAM:SAH ratio, interaction between APC and polyamines, and DNA methyltransferase expression and activity. These may provide firmer evidence as to whether folate and/or methionine will be tumour promoting or tumour protective in this model. The impact of folate and/or methionine on tumour cell

proliferation will ultimately depend on the unique genetics that evolves within each intestinal tumour, which may explain some of the inconsistencies observed between and within experiments.

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